Role of Toll-like Receptor 4 in

Critical Limb Ischaemia

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DECLARATION

I, Ali H. Navi, confirm that the work presented in this thesis is my own, with the guidance of my supervisors, Miss Janice Tsui and Professor David Abraham. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Toll-like receptors (TLR) are key pattern recognition receptors in the innate immune system. In particular, TLR4-mediated immune response has been implicated in ischaemia-induced tissue injury. Mounting evidence supports a detrimental role of the innate immune system in the pathophysiology of skeletal muscle damage in patients with critical limb ischaemia (CLI), in whom patient oriented functional outcomes are poor. The overall aim of this study was to investigate the potential role of TLR4 in skeletal muscle dysfunction and damage in CLI.

The role of TLR4 in ischaemic muscle was studied with three aims: 1) To develop a biobank of human gastrocnemius muscle from stratified patients and to explore TLR4 expression and distribution in these biopsies 2) To investigate the TLR4 pathway and functional consequences of TLR4 inhibition in myotubes derived from human muscle biopsies 3) To evaluate the therapeutic potential of modulating TLR4 signalling in ischaemic muscle using a relevant mouse hind-limb ischaemia model.

TLR4 was found to be expressed in muscle biopsies, with significant upregulation in samples from patients with CLI. *In vitro* studies using cultured human myotubes demonstrated upregulation of TLR4 in ischaemia, with activation of the downstream signalling pathway. Inhibition of TLR4 prior to ischaemia was associated with reduced ischaemia-induced apoptosis. The mouse hind-limb ischaemia model also showed upregulation of TLR4 in

ischaemic muscle. Inhibition of TLR4 was associated with reduced inflammatory cell infiltration and diminished apoptosis in the ischaemic limb.

In conclusion, work presented in this thesis demonstrated that TLR4 is upregulated and activated in ischaemic skeletal muscle in patients with CLI. Modulating TLR4 signalling *in vitro and in vivo* was associated with attenuation of ischaemia-induced skeletal muscle damage. This strategy could be explored further for potential clinical application.

IMPACT STATEMENT

Peripheral arterial disease (PAD) is a progressive condition characterised by atherosclerotic narrowings and occlusions of the lower limb arteries. Its prevalence is growing predominantly due to an ageing population and it is a global health burden. The main treatment options in patients with critical limb ischaemia (CLI), the most severe form of PAD, remain limited to revascularisation or amputation. However, the poor functional outcomes have highlighted the importance of other contributory factors involved in the pathophysiology of the CLI.

We have shown that in skeletal muscle of patients with CLI TLR4 activation occurs and contributes to inflammation and tissue damage which may in turn result in muscle dysfunction. Further we have successfully cultured human skeletal myotubes and demonstrated that TLR4 inhibition attenuates ischaemia-induced skeletal muscle. Finally, we have developed a murine model of hind-limb ischaemia which results in skeletal muscle changes that closely mimics those that occur in patients with CLI. Using this model, we were able to confirm in vivo the role of TLR4 in ischaemia induced muscle damage.

Taken together, our findings suggest that pharmaceutical inhibition of the TLR4 pathway might be of clinical significance in patients with PAD where it could be used as an adjunctive treatment to improve outcomes of treatments at different stages of the disease. TLR4 antagonists are already in

development for use in other areas, making translation to patient benefit feasible in a realistic timescale.

Both the in vitro and in vivo models developed in this thesis would also be useful tools for further research into PAD. The in vitro myotube model would allow drug testing in cells from a relevant patient group whilst the in vivo model would facilitate evaluation of strategies targeting ischaemic muscle changes.

The impact of the work performed in this thesis has so far been demonstrated by presentations at prestigious national and international scientific vascular meetings such as the Vascular Society for Great Britain and Ireland (VS), Society of Academic & Research Surgery (SARS), and Arteriosclerosis, Thrombosis and Vascular Biology (ATVB), and also publications in the relevant peer reviewed journals.

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1.1. Peripheral Arterial Disease

1.1.1. Definition & epidemiology

Peripheral arterial disease (PAD) is a progressive condition characterised by stenoses and occlusions of the peripheral arterial tree [Figure 1]. The prevalence of PAD is growing and is a global health burden (1). Sixteen percent (27million) of the population ≥55 years of age in Europe and North America have PAD. While up to 38% of these patients are asymptomatic, the rest experience some degree of symptoms ranging from mild intermittent claudication (IC) to the most severe form that is critical limb ischaemia (CLI). The European Consensus Document (1989) defined critical ischaemia of the limb as “persistently recurring rest pain requiring analgesia for more than 2 weeks, or ulceration or gangrene of the foot in the presence of an ankle systolic pressure less than 50 mmHg (or a toe pressure less than 30 mmHg in diabetic patients)”. Patients with CLI who do not undergo revascularisation have one-year mortality and amputation rates as high as 54% and 46%, respectively (2, 3).

A recent meta-analysis of 34 studies conducted by Fowkes *et al*. estimated that more than 200 million people are affected by PAD globally, with a relative increase in PAD prevalence of 23.5% during the first decade of the 21st century (4). The economic burden of PAD-related hospitalisation is high, especially as this debilitating condition often leads to multiple hospitalisations (5).

1.1.2. Management options & clinical outcomes

Management options for patients with PAD, who suffer from mild to moderate IC, is mainly aimed at best medical therapy to reduce cardiovascular risk factors and to improve quality of life, whilst in patients with CLI revascularisation is required to salvage the limb.

Despite in current treatments, the outcomes remain poor especially in patients with CLI (6). Outcome reports of lower limb amputations in patients with CLI between 2003 and 2008 in England showed an overall major amputation rate of 5.1 per 100,000 populations with a 16.8% in-hospital mortality rate. 53.3% of patients undergoing major amputation had a revascularisation procedure within the prior 3 years (7). Another population based observational cohort study between 2008 and 2013, showed that the cumulative incidence of major ipsilateral amputation or death in patients with CLI (n=10,617) was 48.8% at 36 months after their first revascularisation procedure (8).

Further, increasing number of studies have demonstrated that functional outcomes of patients with CLI who undergo revascularisation procedures are poor (9, 10). Whilst limb salvage may improve quality of life in some patients, it does not equate to return to premorbid ambulatory or occupational status (11). This suggests that adjunctive treatments in addition to revascularisation are required to improve outcomes.

Physician-oriented outcomes are mainly described as patency rates, limb salvage rates or ulcer healing rates, which do not necessarily translate into improvement from the patient's point of view. Taylor *et al*. reported clinical success using patient-oriented outcome measured after lower limb revascularisation for ischaemic tissue loss. Revascularisation was considered to be clinically successful if each of the following occurred: reconstruction patency until wound healing, limb salvage for 1 year, maintenance of ambulation for 1 year, and survival for 6 months. This study showed only 40.9% overall clinical success rate (n=277; clinical success was 44.3% and 37% in open and endovascular revascularisation, respectively). This study showed that current surgical interventions for CLI are not associated with impressive outcomes (12).

Cieri *et al*. conducted an observational retrospective cohort study to evaluate the functional outcomes in patients with CLI who underwent either

infrainguinal arterial bypass or endovascular revascularisation. They recruited 248 consecutive patients with CLI between 2006 and 2008. Only patients who survived the primary operation / procedure were included. The activities of daily living were measured using the Katz Index (9). The demographics in the sample group were representative of the general population of patients with CLI. During the study period, (mean follow up period of 16.9 +/- 10.75 months from revascularisation) 24 patients (9.7%) underwent major amputation, 46 patients (11.2%) were still in rest pain and 35 patients (14.1%) showed no improvement in tissue loss. Fifty-nine patients (43.2%) required secondary procedures. Physician-orientated outcomes however were good, with 90% limb salvage rate and 82% patency rates at 24 months. The patency rates were higher in patients who had surgical bypasses than in those who had endovascular revascularisation (90% *versus* 77%, P=0.035). Despite good patency rates, the functional abilities failed to improve as expected [Figure 2]. These data suggest that successful revascularisation is not associated with improved functional abilities in patients with CLI (Evidence Level 1b) (Appendix 6).

The impact of PAD on individuals and society is high and deserves greater research focus. Despite progress in vascular and endovascular techniques, outcomes of revascularisation remain poor. This suggests that adjunctive treatments in addition to revascularisation are required to improve outcomes.

1.1.3. Pathology: Atherosclerosis and skeletal muscle changes

The risk factors associated with PAD are determined from large epidemiological studies and are very similar to the risk factors contributing to cerebrovascular disease and ischaemic heart disease. The major risk factors are sex, age, diabetes, hypertension and hyperlipidaemia (13).

PAD is considered to be a set of syndromes resulting from the presence of occlusion or narrowing in the peripheral arterial tree. This is the result of atherosclerosis in most cases.

Endothelial dysfunction is a precursor to atherosclerosis and a key component of PAD. The endothelium actively participates in all stages of atherosclerotic lesion formation. Endothelial dysfunction describes changes in the normal homeostatic control mechanisms of the endothelium that can lead to the development and progression of atherosclerosis (14). In such situations, the endothelial function favours vasoconstriction, thrombus, platelet activation, migration and growth of smooth muscle cells, promotion of vascular inflammation, and oxidative stress. Oxidative stress, which is characterised by increased production of reactive oxygen species (ROS) [Figure 3] appears to be the common underlying factor of the risk factors of endothelial dysfunction and consequent PAD and other atherosclerotic diseases.

Atherosclerosis is described in three stages: early, developing and mature lesions. Early lesions are characterised by areas of lipid deposition, which consist of lipid-filled macrophages and smooth muscle cells in focal areas of the intima. Developing lesions are fibrous plaques, which are firm and covered by a fibromuscular layer. Mature lesions are characterised by calcified fibrotic plaques, which are often symptomatic by either reducing the blood flow to the distal tissue, or by becoming ulcerated and causing thromboembolic events (15). Reduced blood supply in the lower limb results in arterial claudication when the balance between oxygen demand and supply is compromised during exercise. In patients with CLI, this balance is disrupted even at rest causing rest pain and tissue loss.

In 1966, it was reported that patients with PAD who undertake regular exercise can improve their walking distance while mean arterial blood flow in the lower limb remains the same, suggesting mechanisms other than haemodynamic effects (16). Since then, it has been demonstrated that changes within the lower limb skeletal muscle occur in patients with PAD and contributes to the clinical manifestations and poor functional status of these patients (17-19). A number of studies based on light and electron microscopic

assessments of the skeletal muscle in patients with PAD described a myopathy with myofibre degeneration, alteration in the size and shape of the myofibres, shift of myofibre phenotype towards type I fibres, myofibre necrosis and fatty infiltration in patients with IC and CLI (20-22). Koutakis *et al*. quantified the morphological damage to myofibres by investigating gastrocnemius muscle biopsies from patients with PAD who suffered from IC, and showed that the morphological changes in gastrocnemius muscle of patients with PAD are closely linked to calf muscle strength and walking distance (23).

A key element in the process of this myopathy appear to be faulty mitochondria that results in failure in energy regulation, ROS production and apoptosis. It has also been shown that PAD induces inflammation which play a key role in lower limb tissue damage (18). Thus, prevention and treatment of the muscle damage may enable us to improve the outcome of patients with PAD [Figure 4].

1.2. Toll-like Receptors (TLRs)

1.2.1. Innate immune system

The innate immune system is the first line immune response to pathogens. The innate immune cells recognize damage-associated molecular patters (DAMPs) and pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). DAMPs are upregulated in any injury or tissue damage. In case of chronic inflammation, DAMPs, such as high mobility group box 1 (HMGB1) and heat shock proteins (HSPs), play a vital role in the pathogenesis of the disease (24). HMGB1 and HSPs have been shown to be involved in different pathologies such as atherosclerosis and inflammatory bowel disease. HMGB1 has also been shown to play a significant role in ischaemia reperfusion injury in the liver [Table 1] (24). One of the well characterized PRRs are the TLRs (25).

Pathology	DAMP	Effect
Atherosclerosis	Apo CIII-rich VLDL	Stimulated TLR2 dependent monocyte activation and adhesion
Brain injury	HMGB1	Increased cytokines, taste aversion, fever, mechanical allodynia, promotes severity of infarction
Gut inflammation	HMGB1	Increased ileal mucosal permeability and bacterial translocation to mesenteric lymph nodes
Joint disease	FNEDA HMGB1 Tenascin-C	Induced TLR4 dependent transient ankle swelling, cytokine synthesis, synovial inflammation, pannus formation, and induced TLR4 dependent joint inflammation and tissue erosion
Liver injury	DNA HMGB1	During acetaminophen induced cell death induced TLR9 dependent tissue injury, aggravated ischaemic reperfusion injury
Lung injury	HMGB1	Stimulated acute inflammatory injury, neutrophil accumulation, oedema, and cytokine production
Sepsis	HS HMGB1	Induced TLR4 dependent lethality

Table 1 – Piccinini *et al*. described the role of DAMPs in different pathologies (24)

1.2.2. TLR structure

The mammalian TLRs can be categorized as cell membrane receptors (TLR1, TLR2, TLR4, TLR5, and TLR6) or intracellular receptors (TLR3, TLR7, TLR8, and TLR9). Both groups share a similar structure where the cell membrane receptors recognise ligands that are primarily present in the extracellular space whilst the intracellular receptors recognise the ligands that are primarily produced in the intracellular space.

TLRs are type 1 transmembrane proteins with an ectodomain consisting of leucine-rich repeats required to recognize exogenous ligands (PAMPs or DAMPs); a transmembrane domain, which determines cellular localization; and an intracellular domain (TIR) needed for downstream signalling (26).

It appears that homo-/hetero-dimerisation of TLRs and the involvement of different co-receptors and accessory molecules confer specificity to ligand detection and response (24). Most of the TLRs form homodimers, however TLR2 forms heterodimers with TLR1 or TLR6, and TLR4 heterodimerises with TLR6 (25). There is evidence that TLR4 forms a heterodimer with TLR5, and also with TLR1 (27, 28). Furthermore, TLR4 has been shown to form heterodimers with TLR1 in response to exogenous ligands such as lipopolysaccharide (27). Whilst the expression of TLR4 has been shown in skeletal muscle with its upregulation in inflammatory myopathies (29-31), its role in lower limb ischaemia secondary to PAD is unclear. Also, the link between TLR4 and TLR1 has not been investigated in CLI.

1.2.3. TLR ligands

TLRs were initially shown to recognise and respond to conserved components of microbes (32). In addition to binding exogenous ligands derived from the pathogens, TLRs interact with endogenous molecules released from damaged tissues or dead cells and regulate many sterile inflammation processes (25, 26, 32). Endogenous TLR ligands include HSPs, HMGB1 and breakdown products of fibronectin, heparin sulphate and hyaluronic acid, which are exposed during cellular injury and death and extracellular matrix degradation (33). By responding to these DAMPs, TLRs have been implicated in a range of inflammatory and autoimmune conditions (34). Figure 5 shows some of the known exogenous and endogenous ligands of TLRs (25, 33).

1.2.4. TLR downstream signalling

Downstream signalling starts upon the recognition of DAMPs or PAMPs by TLRs leading to the recruitment of adaptor proteins such as MyD88 and TRIF. Two downstream signalling pathways are involved: the MyD88-dependent and TRIF-dependent pathways. Each pathway culminates in the activation of

specific transcription factors such as NFkB, JNK and MAPK, which are required for inflammatory gene transcription. This in turn results in the release of a wide range of cytokines and inflammatory markers such as IL1, 6 and 8, TNFα, INFα and INFβ (25). Apart from TLR3, which only utilises the TRIF-dependent pathway resulting in INFα secretion, all TLRs recruit MyD88. TLR4 recruits both MyD88- and TRIF-dependent pathways. The specific TLR activated, its cellular localisation and the activating ligand involved all appear to determine which signalling pathway is activated. Heterodimerisation together with the involvement of accessory molecules and co-receptors such as MD-2 confer increasing specificity of these receptors to different ligands (35) [Figure 6].

1.2.5. TLRs, chronic inflammation and autoimmunity

The immune response mediated by TLRs is protective in most cases as pathogens are destroyed before they harm the host. However if inflammation becomes chronic, excessive tissue damage may occur (24, 34) [Figure 7]. There is increasing evidence that TLRs play a key role in developing inflammatory responses in conditions such as atherosclerosis and cardiac muscle ischaemia. TLRs have also been implicated in cerebral, renal, liver and myocardial ischaemia by contributing to the inflammatory process and the subsequent tissue injury in animal models (34, 36, 37). Previous work in our lab has shown that TLRs contribute to the inflammatory process in ischaemic muscle injury *in vitro* (38).

1.2.6. Therapeutic role of TLR modulation in cardiovascular dysfunction

Whilst the role of TLRs in PAD has not been explored, there is increasing evidence that they play a role in cardiovascular diseases and suggesting that manipulation of TLR pathways may be of benefit (39). Understanding the molecular structure of TLRs, the downstream signalling pathways and the role of inappropriate TLR stimulation has encouraged the development of specific

TLR agonists and antagonists for therapeutic purposes (40, 41). Both TLR activation and inhibition have been shown to be of benefit in various conditions by reducing the tissue inflammatory response. The effects of TLR agonists and antagonists appear to be dependent on the timing of administration of these modulators during the course of the pathology (42) [Figure 8].

1.2.6.1. Cardiac dysfunction (Table 2)

Ischaemic Heart Disease

TLR2 and TLR4 are activated during myocardial ischaemia and result in NFkB activation with the subsequent release of inflammatory cytokines, which may cause injury to myocardium. TLR2 or TLR4 deficiency attenuates myocardial inflammation, reduces infarct size, and preserves ventricular function after transient ischemic injury (43-47). The potential benefits of TLR antagonists in myocardial ischaemia have therefore been explored.

TLR antagonists: OPN-301 is a mouse IgG1 antibody that selectively inhibits TLR2 activity in mouse, pig, monkey, and humans. Arslan and colleagues showed that OPN-301 reduces myocardial I/R injury in a mouse model by downregulating NFkB signalling and therefore reducing leukocyte infiltration and apoptosis (43).

The TLR4 antagonist, Eritoran, is an analogue of the lipid A portion of the bacterial derived LPS with long pharmacokinetics but short pharmacodynamics half-life. In a mouse model of myocardial ischaemia, Eritoran has been found to be protective by modifying the immune response and reducing infarct size (48). Emotional stress can contribute to the pathophysiology of coronary atherosclerosis by accelerating the plaque formation or causing an acute cardiac event in patients with advanced coronary atherosclerosis (49, 50). In an animal model of chronic stress,

Eritoran inhibited NFkB activity and reduced stress-related myocardial injury (51).

TLR agonists: Interestingly, TLR agonists have also been found to be beneficial in models of myocardial ischaemia. Lipopolysaccharide (LPS) is a TLR4 agonist that is a major structural component of the outer wall of all Gram-negative bacteria. It has protective effects in cardiac ischaemia (Zacharowski et al., 2000, Ha et al., 2008) via TLR4-induced MyD88-dependent pathway activation (52). The cardioprotective effect of LPS appears between 12-24 hours after LPS administration (53). TLR4 also has cardioprotective effects by enhancing the production of inducible nitric oxide synthase (iNOS) (54, 55). Wang and colleagues demonstrated that activation of the TLR4-mediated MyD88-signalling pathway by LPS led to increased production of iNOS and generation of nitric oxide with improved ventricular function (52). Administration of low doses of TLR2 or TLR4 ligands prior to myocardial I/R is also associated with reduced myocardial damage. In a mouse model, administrating the TLR2 agonist Pam3CSK4, a synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial lipopeptides, prior to ischaemia rendered the myocardium less susceptible to subsequent I/R injury and reduced myocardial damage (56). The exact mechanism of this protective effect is unclear, but it is thought that stimulating TLR with a low dose of its ligand, may precondition or reprogramme the signalling pathway to attenuate the inflammatory response and subsequent tissue damage. Serial stimulation of TLRs is associated with differential regulation of the MyD88-dependent and MyD88-independent signalling pathways (57). The initial low dose TLR ligand promotes signalling via the MyD88 pathway, while the subsequent stimulation results in reduced MyD88-dependent and enhanced MyD88-independent signalling and subsequently, attenuates cytokine responses.

Heart failure

Heart failure is associated with chronic inflammation (58) and upregulation of TLR4 in cardiac muscle (59, 60). Absence of TLR4 prevents the increase in myocyte and cardiac size following pressure overload (61). The acute activation of TLR signalling may be beneficial in the short term, but ongoing tissue damage which results in chronic activation of TLRs can lead to heart failure (62). Both TLR antagonists and agonists have been shown to be protective in heart failure.

TLR antagonists: Eritoran has also been shown to reduce cardiac hypertrophy in a mouse model of aortic constriction by inhibiting a TLR4-mediated inflammatory response (63). Thus, patients with pressure overloaded induced heart failure may benefit from the inhibition of TLR4 signalling. However the pharmacodynamics of Eritoran requires administration by a continuous infusion that would not be practical for the treatment of patients with chronic heart failure.

TLR agonists: Bacillus Calmette-Guerin (BCG) is a well-recognised immunotherapy against tuberculosis (64). BCG has also been used for the treatment of patients with bladder cancer by stimulating the immune response via activation of TLR4 (65). The stimulation of TLR4 by BCG also prevented the development of pressure-induced cardiac hypertrophy and fibrosis in a mouse model of abdominal aortic constriction induced hypertension (66).

Myocarditis

Myocarditis is an inflammatory process involving the cardiac muscle and can be caused by infection, autoimmune diseases or toxic chemicals. Up to 20% of sudden cardiovascular deaths amongst young individuals are caused by myocarditis (67, 68).

TLR antagonists: Coxsackievirus B3 (CVB3) is the major cause of human myocarditis (69, 70). In CVB3-mediated autoimmune myocarditis, TLR4 plays a key role in the production of inflammatory cytokines (71-73) leading to

worsening of the condition and therefore TLR4 inhibition may have therapeutic applications.

Other compounds found to be beneficial in cardiac dysfunction have been found to work via anti-TLR mechanisms (42) [Table 2].

Compound	Mechanism	Application	Reference
Pam3CSK4	TLR2 agonist	Cardiac ischaemia	Mersmann 2010
OPN-301	TLR2 antagonist	Cardiac ischaemia	Arslan 2010
LPS	TLR4 agonist	Cardiac ischaemia	Wang 2011a
BCG	TLR4 agonist	Cardiac hypertrophy	Liu 2008
Eritoran	TLR4 antagonist	Cardiac ischaemia	Shimamoto 2006
Valsartan	TLR4 inhibitor	Cardiac ischaemia	Yang 2009
Atorvastatin	TLR4 inhibitor	Cardiac ischaemia	Chansrichavala 2009
Losartan	TLR4 inhibitor	Cardiac failure	Ogawa 2011
Fluvastatin	TLR4 inhibitor	Cardiac failure	Folden 2008
Cinnamaldehyde	TLR4 inhibitor	Myocarditis	Ding 2010
Geldanamycin	TLR4 inhibitor	Myocardial inflammation	Barber 2008

Table 2 - List of compounds shown to modify TLRs in cardiac dysfunction

1.2.6.2. Vascular dysfunction

Cerebrovascular injury (Table 3)

Cerebrovascular diseases refer to a group of brain dysfunctions induced by disease of the blood vessels supplying the brain and include ischaemic and haemorrhagic stroke. TLRs play a key role in the post-ischaemic inflammatory response (IL1, IL6, TNFα) in the brain. In particular, TLR2 and TLR4 activate the inflammatory response and apoptotic pathways (74). TLR2 is mainly expressed in microglia (75) and is activated by endogenous ligands such as HMGB1 and HSPs (76). Intracerebral haemorrhage is responsible for up to 20% of all strokes (77). TLR4-mediated NFkB activation plays an important role in the inflammatory response and cell apoptosis in intracerebral haemorrhage (78, 79). By activating the acute inflammatory response, TLR4 also contributes to the pathogenesis of early brain injury in subarachnoid haemorrhage, the most disabling feature of aneurysmal subarachnoid haemorrhage (80, 81). TLR antagonists may be of benefit in patients with stroke by reducing the inflammatory response in the brain tissue.

TLR antagonists: T2.5, a monoclonal antibody against the extracellular domain of TLR2, has been shown to inhibit TLR2-mediated inflammatory responses in murine and human macrophages (82). In a mouse model of transient brain ischaemia, T2.5 reduced inflammatory cell accumulation and neural death with tendency towards a reduction in infarct size (83).

The TLR4 antagonist, Eritoran, mimics the LPS structure and is therefore too large to pass through the normal blood-brain barrier. However, following a stroke, the blood-brain barrier is compromised which may allow the passage of larger molecules such as Eritoran and this may allow further studies to investigate the role of Eritoran in cerebral infarction. (84).

TLR agonists: Despite the increasing number of studies indicating that TLR inhibition may have a potentially protective role in brain ischaemia there is also evidence to suggest that administration of the TLR ligands prior to the

brain ischaemia is also neuroprotective *in vivo* (85). Thus, high risk patients may benefit from prophylactic TLR agonists prior to the potential brain ischaemia. During major cardiac surgery, patients are exposed to I/R injury of major organs such as the brain and kidneys (86-88). Further, up to 50% of patients undergoing coronary artery bypass graft, experience intra-operative focal or global brain ischaemia (89).

Administration of Pam3CysSerLys4 (Pam3CSK4), a specific TLR2 agonist (90), prior to brain ischaemia resulted in significant reduction in infarct size and mortality rate in a mouse model of cerebral I/R injury (91).

Unmethylated cytosine guanine rich DNA oligonucleotides (CpG ODNs) which are synthetic TLR9 agonists, have been shown to be safe in a number of human clinical trials as a vaccine adjuvant (92). Administration of low dose CpG ODN or LPS prior to stroke in a mouse model of cerebral ischaemia, has protective effects against neuronal ischaemic damage (93). This protective effect is thought be the result of activation of TLR4 and TLR9 and the subsequent downstream signalling pathway that leads to activation of interferon regulatory transcription factor (IRF) (93). IRFs are key modulators of type I interferon gene regulation. Further, prophylactic LPS has also been shown to reduce ischaemic injury in hippocampal neurons in a pig model of cardiopulmonary bypass (94)**.**

Other compounds found to be beneficial in cerebrovascular dysfunction have been found to work via anti-TLR mechanisms (42) [Table 3].

Compound	Mechanism	Application	Reference
Pam3CSK4	TLR2 agonist	Brain ischaemia	Hua 2008
T2.5	TLR2 antagonist	Brain ischaemia	Meng 2004
Baicalin	TLR2 and TLR4 inhibitor	Brain ischaemia	Tu 2011

Oxymatrine	TLR2 and TLR4 inhibitor	Brain ischaemia	Fan 2009
LPS	TLR4 agonist	Brain ischaemia	Stevens 2011
Progesterone	TLR4 inhibitor	Brain ischaemia	Wang 2011
Ginkgobiloba	TLR4inhibitor	Brain ischaemia	Hu 2011
Picroside 2	TLR4 inhibitor	Brain ischaemia	Guo 2011
CpG ODN 1826	TLR9 agonist	Brain ischaemia	Stevens 2011

Table 3 - List of compounds shown to modify TLRs in cerebrovascular dysfunction

Ischaemic reperfusion injury (Table 4)

I/R injury is an antigen independent inflammatory process characterized by activation of the innate immune system (95). While the initial ischaemia causes tissue injury, reperfusion can further enhance the tissue response.

Ischaemic reperfusion liver injury

Liver ischaemia is common during hepatectomy and liver transplantation. The ischaemia-induced inflammatory response contributes to the liver damage. TLR3 and TLR4 have been found mediating the inflammatory response in liver ischaemia. TLR4 induces the inflammatory response in liver I/R injury by activating transcription factors such as NFkB, JNK and MAP kinases (96-101).

TLR agonists: Administration of TLR ligands prior to ischaemia, has been shown to be protective during the reperfusion period by suppressing and redirecting the tissue response (102, 103). The predictability of liver I/R injury during a liver transplant encourages the application of preventive strategies such as pre-treatment with TLR ligands prior to I/R injury to minimize the potential liver damage (104-107). In a mouse model of liver ischaemia, pre-treatment with LPS protected the liver by activating the TLR4-mediated IRAK

signalling pathway during the pre-ischaemic period with subsequent suppression of NFkB and JNK during reperfusion (108). Further, LPS has also been shown to be protective in a mouse model of liver I/R injury by suppressing TLR4 signalling through early induction of suppressor of cytokine signalling 1 (SOCS-1), a negative regulator of cytokine signalling (109).

Ischaemic reperfusion renal injury

Peri-operative acute kidney injury is a well-recognized complication following major cardiothoracic and vascular surgeries (110-112). Changes in haemodynamics may cause renal tubular injury followed by activation of the immune system (113). Both innate and adaptive immune responses contribute in renal I/R injury, a well-recognised cause of acute kidney failure (114, 115). Activation of the immune system leads to endothelial and epithelial cell damage. The inflammatory process begins within minutes of reperfusion through innate immune response and is followed by a late adaptive response that manifests within a few days. (115, 116). TLR4 is normally expressed in the collecting ducts, loop of Henle, proximal and distal tubules (117). Considering that I/R injury is associated with upregulation of TLR4 in kidney epithelial cells where they contribute to acute kidney injury (118, 119), TLR4 antagonists may have therapeutic role in protecting the kidneys in renal I/R injury.

TLR antagonist: Eritoran has been shown to have protective effects on the kidney in renal I/R injury. Liu and colleagues demonstrated that administration of Eritoran reduced the inflammatory gene expression and enhanced the morphological features in the kidney in a rat model of renal I/R injury (120).

TLR agonists: Pre-treatment with TLR ligands, has also been shown to be protective in renal I/R injury. Polyinosinic polycytidylic acid (poly-IC), a TLR3 agonist, is a synthetic double stranded RNA. In order to improve the pharmacokinetics of poly-IC, it is stabilized with poly-L-lysine and carboxymethylcellulose moieties. This version of poly-IC (poly-ICLC) has

been shown to be safe in humans in a number of conditions including viral infection, multiple sclerosis and cancer (121, 122). Pre-treatment with poly-ICLC had a protective effect in mouse models of renal and cerebral I/R injury *in vitro* and *in vivo*, by reducing the release of inflammatory cytokines including IL1, IL6, IL12, TNFα and INF and also decreasing infarct size (123). Flagellin, a TLR5 agonist is a protein that arranges itself into a hollow cylinder to form the filament in bacterial flagellum. Pre-treatment with CBLB-502, a pharmacologically optimized Flagellin derivate has been shown to be protective in I/R injury by decreasing the accumulation of reactive oxygen species (ROS). In a mouse model of renal I/R injury, pre-treatment with CBLB502 inhibited neutrophil infiltration during the reperfusion period (124). In addition, administration of CBLB502 immediately after reperfusion was also protective in renal I/R injury. Thus, these protective properties can be of potential benefit during the reperfusion of transplanted organs.

Ischaemic reperfusion lung injury

Ischaemic reperfusion injury in the lung can be characterized by non-specific alveolar damage and lung oedema that is followed by systemic hypoxia (125). In particular, TLR4 plays a key role in the pathogenesis of I/R injury by increasing microvascular permeability, activating the NFkB signalling pathway and inducing apoptosis in lung tissue (48, 126, 127).

Other compounds found to be beneficial in I/R injury have been found to work via anti-TLR mechanisms (42) [Table 4].

Compound	Target	Application	Reference
Poly-ICLC	TLR3 agonist	Renal ischaemia Brain ischaemia	Packard 2012

Lipopolysaccharide	TLR4 agonist	Liver I/R injury	Sano 2010
Eritoran	TLR4 antagonist	Renal I/R injury	Liu 2010
Oestrogen	TLR4 inhibitor	Lung I/R injury	Shih 2011
Dexmedetomidine	TLR4 inhibitor	Renal ischaemia	Gu 2011
Melatonin	TLR3&4 inhibitor	Liver I/R injury	Kang 2011
CBLB502	TLR5 agonist	Renal I/R injury	Fukuzawa 2011

Table 4 - List of compounds shown to modify TLRs in I/R injury

Modulating TLRs by both stimulation and inhibition of TLR signalling has been shown to have therapeutic potential in various models of cardiovascular diseases. TLR antagonists have clearly been shown to be protective by directly inhibiting inflammatory gene transcription and reducing secretion of inflammatory cytokines in several conditions. However, preconditioning with low dose TLR agonists may also be an effective strategy to protect tissue against subsequent detrimental insults such as reperfusion injury. This underscores the importance of administering specific TLR agonists and antagonists during the relevant phase of the pathological process in order to achieve an optimal balance of TLR activation and inhibition. Further translational studies are required to improve the practicality of TLR modulation in cardiovascular diseases.

1.2.7. TLR4 and ischaemic-induced injury

TLRs seem to be involved in a range of diseases associated with vascular dysfunction and ischaemic tissue damage, in particular TLR4, its involvement in PAD and resultant muscle damage is likely.

TLR4 has been shown to be important in animal models of ischaemia: TLR4 deficiency protected against renal ischaemia and reduced tubular damage

(128), reduced myocardial infarct size and protected against focal cerebral ischemia (129). Preliminary work in our lab has shown that TLR4 is upregulated in gastrocnemius muscle biopsies obtained from patients with CLI compared to patients with no PAD (38). Discovering the role of TLR4 stimulation in inflammation has led to the development of specific TLR4 antagonists for therapeutic purposes. Eritoran, a TLR4 antagonist, has been trialled to assess its efficacy in severe sepsis (40). In an animal model of myocardial ischaemia, Eritoran reduced ischaemic injury and inflammation (48). Pharmacological inhibition of TLR4 may therefore also be of benefit in CLI. Results from this study will increase our knowledge on the pathophysiology of PAD, in particular the mechanisms underlying ischaemic muscle damage. Further, the potential of targeting TLR4 signalling to reduce ischemia-induced myopathy will be demonstrated. Ultimately, this may lead to novel treatments to improve outcomes following revascularisation in patients with CLI and/or prevent worsening in those with non-reconstructable disease. Targeting the TLR4 pathway may also prevent progression of the myopathy in patients with earlier disease. Since TLR4 antagonists are already in clinical trials for other indications, this may translate into patient benefit in the near future, leading to improved outcomes and better quality of life.

1.3. Aims & Hypothesis

The overall aim of this study was to investigate the potential role of TLR4 in skeletal muscle dysfunction in CLI.

Objectives:

The objectives were:

1) To investigate expression, distribution and potential activation of TLR4 pathway in critically ischaemic human skeletal muscle

2) To study the functional consequences of TLR4 activation in ischaemic myotubes *in vitro*
3) To assess the effect of TLR4 inhibition in a mouse model of hind limb ischaemia

Hypothesis:

We hypothesized that chronic activation of TLR4 by endogenous ligands released by damaged cells within ischaemic skeletal muscle triggers an amplifying cascade of downstream pathological mechanisms leading to further inflammation and tissue damage. Identification and understanding of early key events in the cascade may allow novel therapies to be developed to prevent progressive muscle damage. In particular, modulating TLR4 signalling *in vivo* may attenuate skeletal muscle damage [Figure 9].

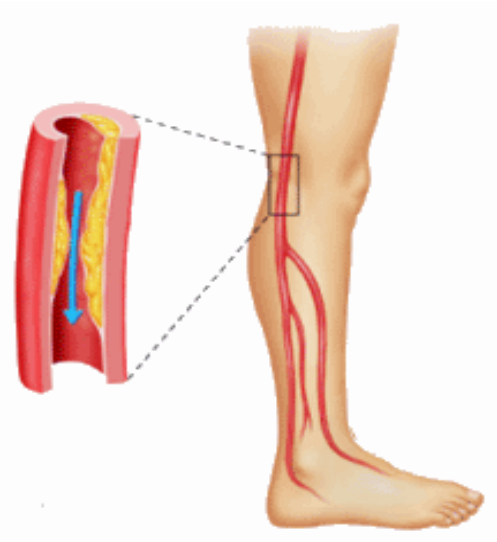

Figure 1 – Demonstrating a stenosis in peripheral arterial tree

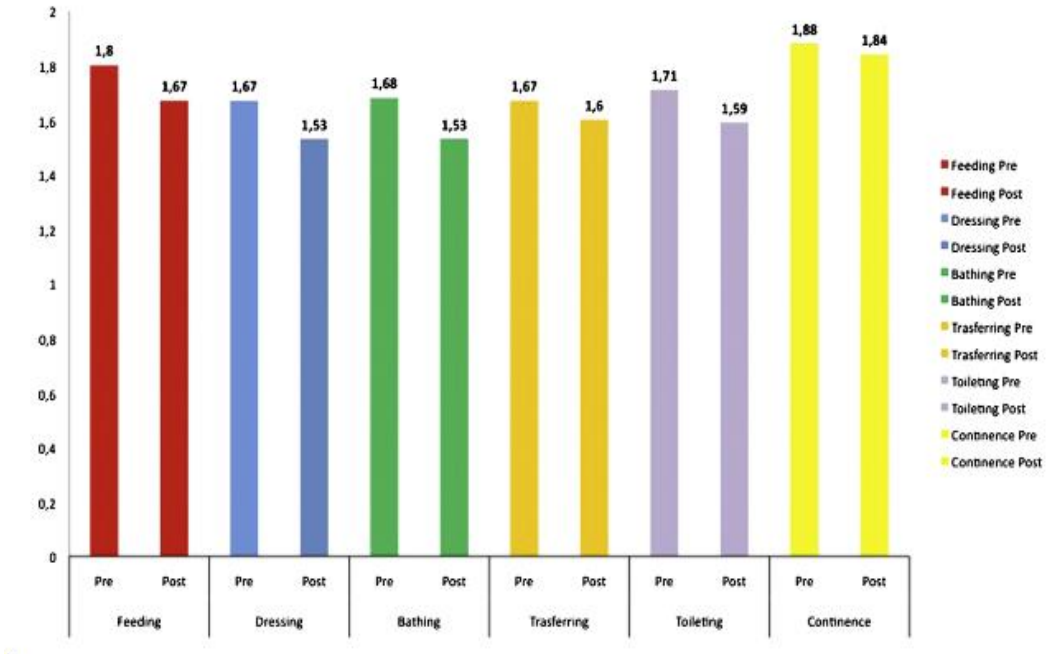

Figure 2 - Katz Index, pre and post revascularisation (9)

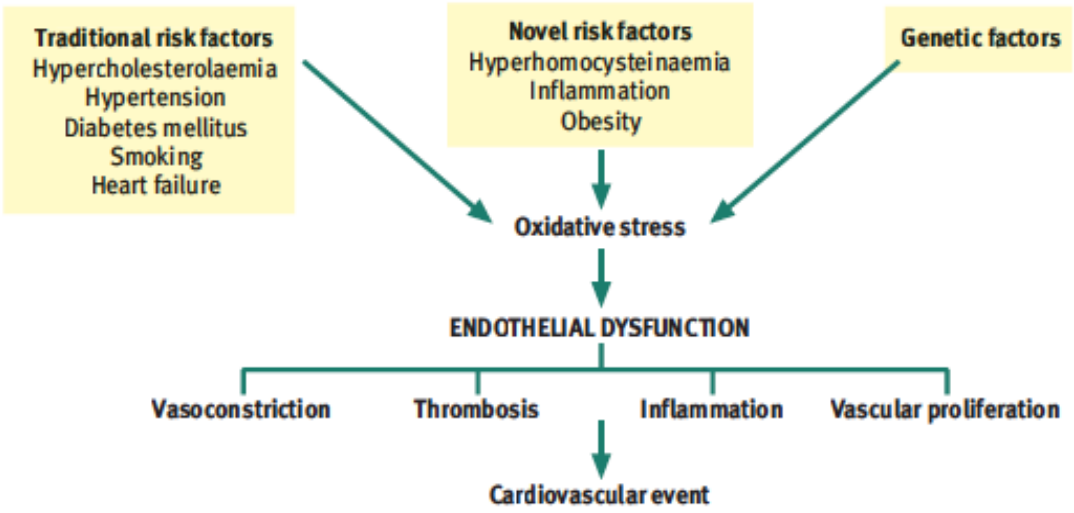

Figure 3 – Risk factors for endothelial dysfunction (14)

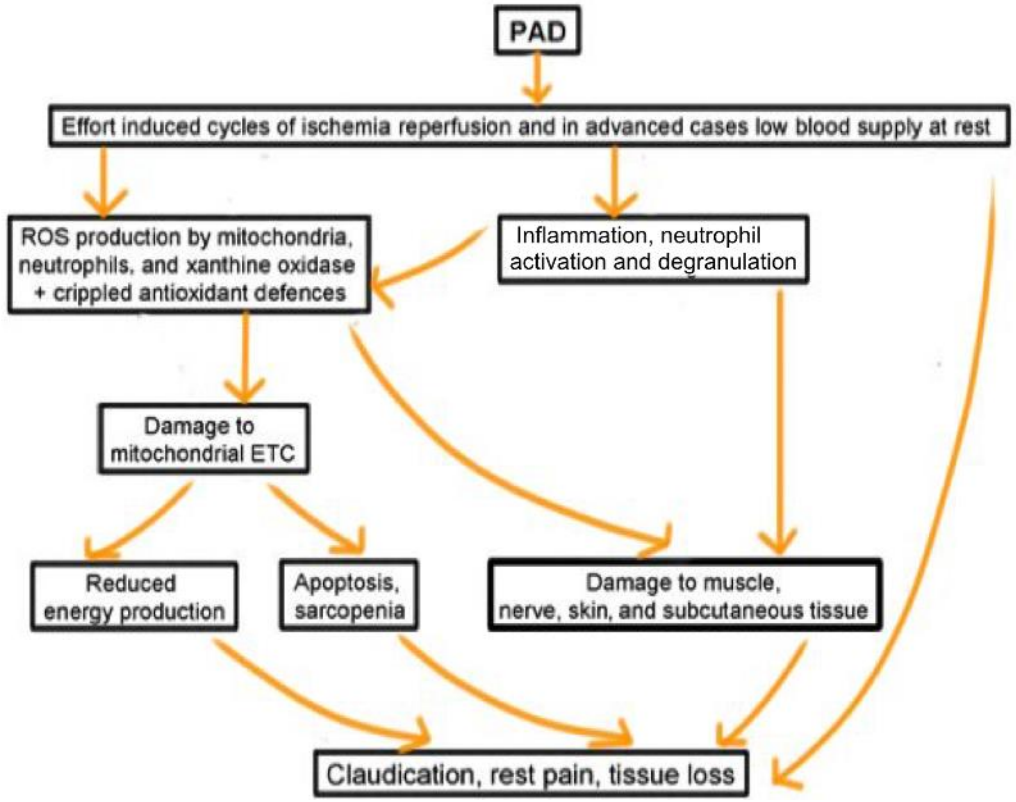

Figure 4 – End organ damage in patients with PAD (18)

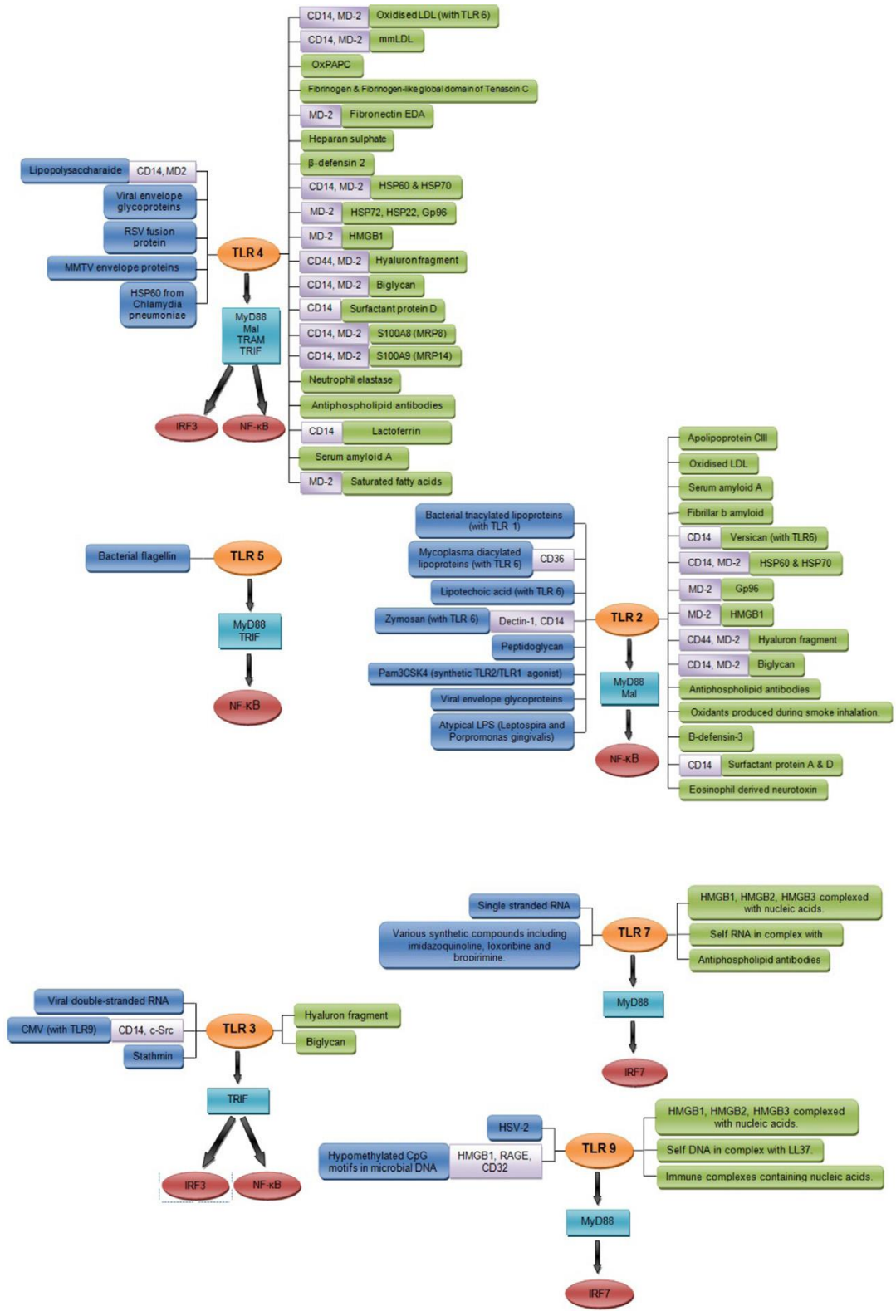

Figure 5 - Exogenous and endogenous ligands of TLRs (25)

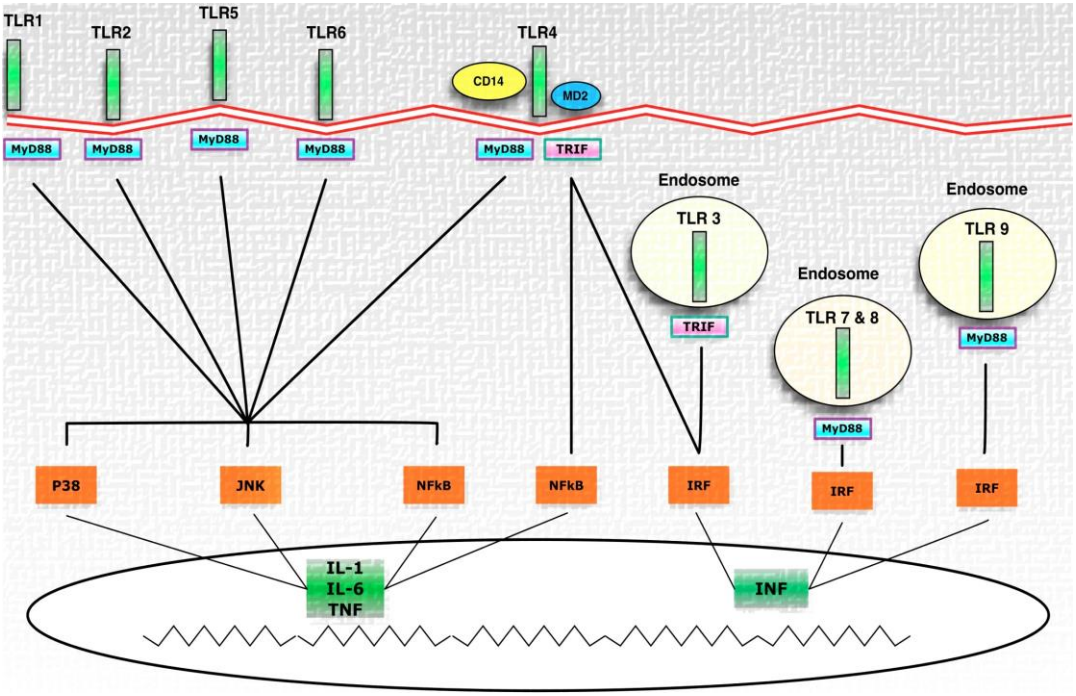

Figure 6 - The TLR signalling pathway

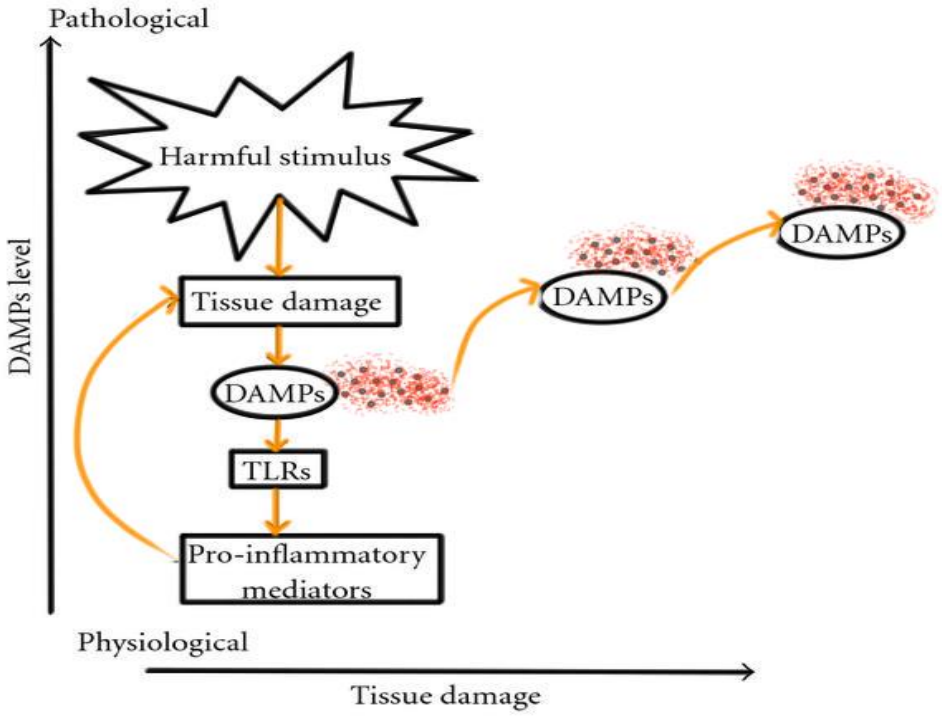

Figure 7 – Unnecessary tissue damage as a result of chronic TLR activation (24)

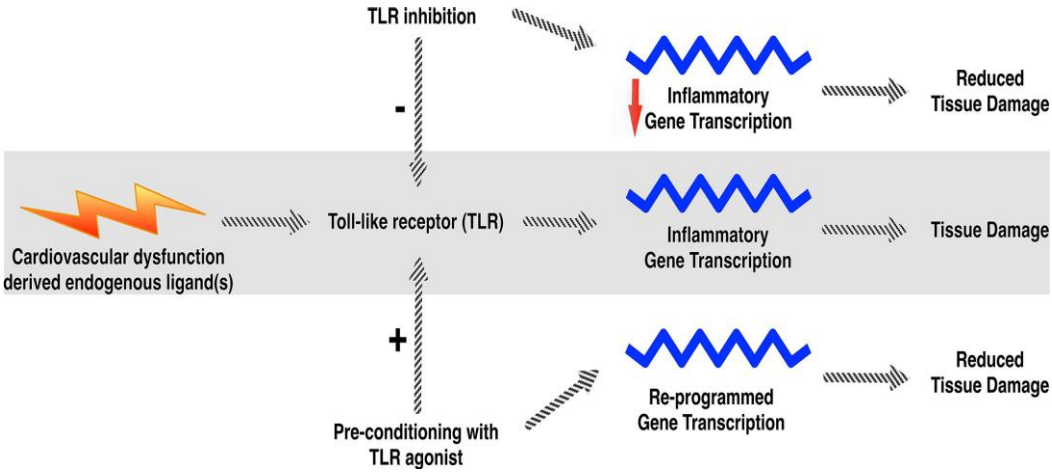

Figure 8 - The effect of TLR modulation (42)

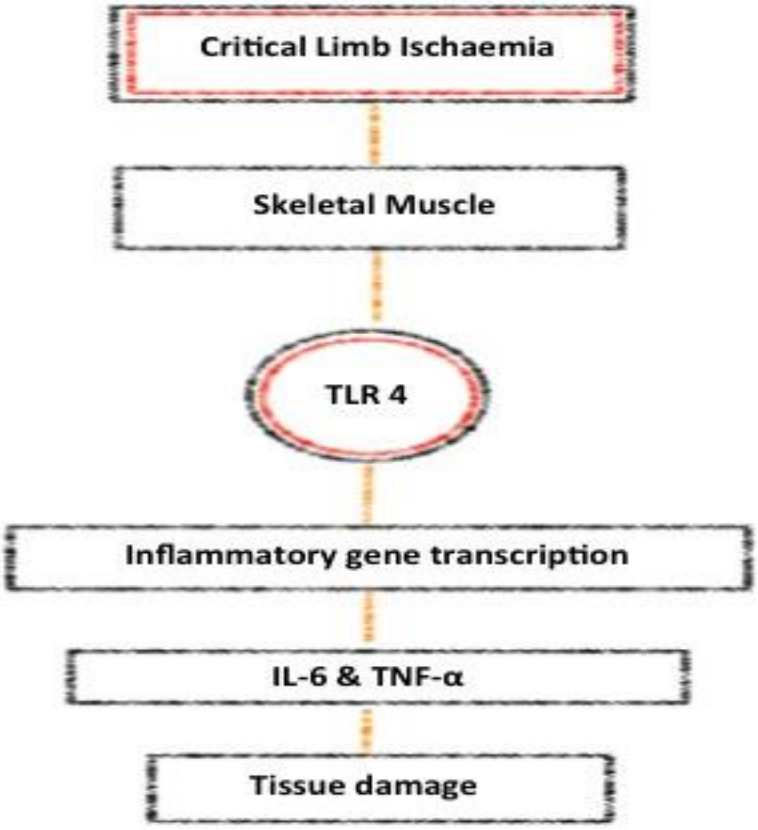

Figure 9 – The potential involvement of TLR4 in ischaemia-induced skeletal muscle damage in patients with CLI

CHAPTER 2: METHODS AND MATERIALS

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2.1. Human tissue collection & preparation

2.1.1. Ethics and patient groups

Local ethics committee approval (REC reference: 29-2000; Royal Free and Medical School Local Research Ethics Committee) was obtained to collect gastrocnemius biopsies from patients undergoing major lower limb amputation for CLI (ischaemic group) and from patients with no PAD undergoing saphenous vein harvesting for coronary artery bypass surgery (control group). As patients in both groups shared similar risk factors, all patients in the control group were reviewed by taking full histories and performing clinical examinations to identify and exclude the ones with underlying PAD. Baseline data of the 2 groups were collected as follows: sex, age, smoking history, eGFR, presence/ absence of hypertension, diabetes, hyperlipidaemia, claudication and ABPI. Patients were provided with information sheets regarding the project and written consent was taken from those who agreed to participate (Appendix 5).

2.1.2. Gastrocnemius muscle biopsy

Muscle biopsies taken from the medial head of gastrocnemius muscle, were cut into smaller pieces and stored as follows:

1) Tissue was placed in 10% formalin and stored at room temperature to be used for immunohistochemistry. 2) Tissue was placed immediately into Eppendorfs in liquid nitrogen and then transferred to -80°C to be used for western blot analysis and ELISA. 3) Tissue was placed immediately into culture medium for isolation of myoblasts to be used in cell culture experiments. 4) Tissue was placed in Eppendorfs containing RNAlater, stored at 4°C overnight and then transferred to -80°C within 24 hours to prevent molecular destruction. These samples were used for RNA experiments.

In order to allow blind analysis, all samples were labelled with serial numbers prior to storage. The consent forms were placed in the allocated secure place after tissue collection.

2.2. Western blotting

2.2.1. Muscle homogenates

Snap frozen samples, stored at -80°C, were cut into smaller fragments and placed in pre-labelled 2ml Eppendorfs which were filled with 20µl of lysis buffer (200µl Triton X100; 1600µl RIPA buffer with added protease inhibitor; Thermo Scientific Pierce, UK) per milligram of tissue. The tissue lyser machine (TissueLyser II, Qiagen) was programmed for 3 cycles of 3 minutes at 30 Hz to achieve adequate lysis. This was followed by centrifugation of the samples at 13000 rpm for 5 minutes.

The BCA (bicinchoninic acid) protein assay kit (Thermo Scientific Pierce) was used to measure total protein concentrations of samples. Standard proteins of known concentration (Bovine serum albumin, 10µl per well) and protein samples of unknown concentration (10µl per well) were added into 96 well plates together with the mixed reagents (200µl per well). The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In the second colour development step, BCA reacts with the reduced (cuprous) cation which was formed in step one. The plate was incubated for 30 minutes at 37°C and then read at light absorbance of 562 nm to calculate the standard curve and to estimate the protein concentration in the samples [Figure 10].

The final concentration of the proteins was adjusted to about 2000µg per millilitre with RIPA buffer. Once the protein concentration was confirmed, phosphatase inhibitor (1µl per 1mg of protein; Thermo Scientific Pierce) was added to each Eppendorf to prevent phosphorylation of the transcription

proteins. The samples were exposed to 95°C for 5 minutes before transferring back to -80°C for storage.

2.2.2. Preparation of cell lysates

Human myotubes were cultured in 6 well plates. Plates were washed thoroughly with Phosphate Buffered Saline (PBS; Life Technologies, UK). Lysis buffer (120µl RIPA with added protease inhibitor, Thermo Scientific Pierce) was added to each plate and mixed using a cell-scraper. Cell lysate contents were placed into labelled Eppendorfs and continually aspirated and expelled with a fine needle for 2 minutes to enhance cell lysis.

The BCA protein assay kit was used as described earlier to determine protein concentration. Once the protein concentration was confirmed, phosphatase inhibitor (1µl per 1 milligram of protein; Thermo Scientific Pierce) was added to each Eppendorf to prevent dephosphorylation of transcription proteins. The samples were exposed to 95°C for 5 minutes before transferring to -80°C for storage.

2.2.3. Electrophoresis, transfer and developing

Western blotting is a semi-quantitative analysis of a specific protein within a heterogeneous mixture of proteins. This technique facilitates the detection and comparison of a specific protein expression between different samples (130). Polyacrylamide gel electrophoresis (PAGE), describes a technique used to separate macromolecules such as proteins according to electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule. In order to separate the proteins of interest (protein sizes varied between 15 and 100 kDa), 10% Tris-Glycine gel was made of resolving gel (16.7ml of 30% Acrylamide; 12.5ml of 1.5 M Tris PH=8.8; 0.5ml of 10% Sodium Dodecyl Sulphate; 19.8ml of dH2O; 0.5ml of 10% Ammonium Persulphate; 0.02ml of TEMED) and stacking gel (30% Acrylamide; 0.5 M Tris PH=6.8; 10% SDS; dH2O; Ammonium Persulphate; TEMED). Sodium

Dodecyl Sulphate (SDS) is an anionic detergent that linearizes proteins by imparting a negative charge (131). The gels were wrapped in wet towel and stored in the fridge for no longer than 2-3 days before use.

Denatured protein samples (thawed adequately and centrifuged at 13000rpm for 5 minutes) were loaded onto 10% Tris-Glycin gels. Each lane was loaded with total volume of 15µl (10µl / 20µg of protein sample and 5µl of laemmli buffer). The laemmli buffer (1 M Tris PH=6.8 / 0.4% SDS (2.4ml), 100% Glyceron (4ml), 0.01% Bromphenol blue (2mg), dH2O (2.8ml) and B-Mercaptoethanol (1ml)) is the standard loading buffer that was first described in 1970 (132). One lane in each gel was loaded with 10µl protein ladder (SeeBlue Plus, Invitrogen).

The loaded gels were placed into the central chamber of an XCell Sure Lock electrophoresis device [Figure 11] and the central and outer chambers were filled with running buffer (100ml of 10 X TrisGlycine Gel diluted in dH_2O into total volume of 1 litre). The gels were run at 125V until the dye reached the bottom of the gels (1.5 – 2 hours). The electroblot was prepared by soaking the sponges in transfer buffer (150ml of 15% Methanol and 40ml of 25 X TrisGlyc transfer buffer (Novex, Life Technologies, Paisley, UK) diluted with dH_2O up to 1 litre) for 1 hour. The blotting stack was prepared by expelling air bubbles from the sponges by rolling gently with a centrifuge tube, and the nitrocellulose membrane (Hybond-C extra; GE Healthcare Life Sciences, Buckinghamshire, UK) was sandwiched in the following order: sponge - paper filter - gel - membrane - paper filter – sponge. The blotting stack was placed in the central chamber of blotting module device which was filled with transfer buffer. The outer chambers were filled with cold dH_2O and the proteins were transferred onto nitrocellulose membrane at 30V for 90 minutes.

In order to block non-specific binding sites, the membranes were blocked with 5% milk solution (5g Marvel Milk Powder, 100ml of Phosphate Buffered

Saline (PBS) / 0.1% Tween 20) for 2 hours. This was followed by incubation of the membrane at 4°C with the primary antibodies shown in Table 5.

Antibodies	Suppliers	Concentrations
TLR1	ab37068; Abcam	1:1000
TRIF	ab13810; Abcam	1:1000
TLR4	ab22048; Abcam	1:500
HSP70	sc-373867; Santa Cruz	1:1000
HSP60	sc-136291; Santa Cruz	1:1000
MyD88	ab2064; Abcam	1:1000
P-P38	D3F9; Cell Signalling	1:1000
P38	9212; Cell Signalling	1:1000
JNK	9252; Cell Signalling	1:1000
P-JNK	9251; Cell Signalling	1:1000
NFkB	C22B4; Cell Signalling	1:1000
P-NFkB	93H1; Cell Signalling	1:2000
Cleaved Caspase-3	9661; Cell Signalling	1:1000
β tubulin loading	ab4074; Abcam	1:1000

Table 5 – List of the primary antibodies

The membranes were then washed and incubated with horseradish peroxidase-linked corresponding secondary antibody (Anti-Rabbit, Anti-Goat or Anti-mouse; Abcam) at room temperature. Both primary and secondary antibodies were diluted in 5% milk solution. The membranes were developed using chemiluminescent substrate (Amersham ECL Plus™ Western Blotting Detection Reagent; GE Healthcare Life Sciences) and the blots were developed against photographic film (Amersham Hyperfilm ECL, GE

Healthcare Life Sciences). The photographic films were exposed to the nitrocellulose membranes in the dark chamber for varying durations from 1 to 30 minutes to facilitate the best exposure onto the films. The films were then placed straight into the developing and fixing solutions. Band intensities were determined by densitometry using computer software ImageJ 1.45s (US National Institutes of Health, Bethesda, Maryland, USA). The membranes were washed with PBS / 0.1% Tween 20 and re-used to measure protein loading (β-tubulin loading control).

2.3. Immunohistochemistry

Immunohistochemistry (IHC) is a technique to demonstrate the presence and location of specific proteins in tissue sections. Although this technique is less sensitive quantitatively than western blot and ELISA, it provides more information on the distribution and location of the protein.

Antibodies that recognize the specific target antigens achieve immunohistochemical staining. The antibody-antigen complex is then visualized using either chromogenic detection or fluorescent detection (133).

The human and mouse skeletal muscle biopsies were fixed in 10% formalin and embedded in paraffin, then cut by microtome to approximate 10-micron thickness and affixed onto slides. Slides were left at room temperature overnight to dry out.

Before proceeding with the staining, the slides were washed with xylene and ethanol to deparaffinise and rehydrate the sections respectively.

Fixation in formalin results in formation of methylene bridges, which mask antigen sites, and therefore an antigen retrieval step is required to overcome this problem. In order to perform antigen retrieval, the slides were placed in pre-warmed citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20 PH=6.0) for 5 minutes on full power (P100) in the microwave. After antigen retrieval,

the slides were washed generously with PBS and placed in a humidified chamber [Figure 12].

2.3.1. Biotinylated immunohistochemistry – Paraffin embedded sections

Slides were incubated with blocking solution (10% normal serum of the species that the secondary antibody was raised in with 1% BSA in PBS / 0.1% Tween 20) for 2 hours at room temperature to prevent non-specific binding of the secondary antibody.

The primary antibody (TLR4; 1:100; abcam) was then applied, diluted in 1% BSA in PBS / 0.1% Tween 20, and incubated overnight at 4°C.

Blocking of the endogenous peroxidase was performed by incubating the slides in 0.3% H_2O_2 in PBS for 15 minutes. Then, the biotinylated HRP-conjugated secondary antibody (anti-mouse; 1:200; ab675; abcam) was applied, diluted in 1% BSA in PBS / 0.1% Tween 20, and incubated for 30 minutes at room temperature.

This was followed by applying Avidin and Biotinylated horseradish peroxidase macromolecular Complex (VectaStain Elite ABC kit; Vector Laboratories) onto the slides for an additional 30 minutes to amplify the signal. Then, the horseradish peroxidase is visualized by the development of the peroxidase substrate, 3,3-Diaminobenzidine (DAB), which produces a brown colour.

Further counterstaining with haematoxylin (blue) was performed to observe tissue and cell morphology. The slides were incubated in Mayer's haematoxylin for 5 minutes, and then briefly exposed to acid alcohol followed by incubation in blueing solution for 5 minutes. After further wash with PBS, the slides were placed in a rack and washed with ethanol and xylene to dehydrate the sections. The images were viewed using a microscope (Axioscop 2; Carl Zeiss Micro-imaging).

2.3.2. Fluorescent immunohistochemistry – Paraffin embedded sections

In order to examine the co-distribution of two different antigens in the same sample, double labelled fluorescent staining was carried out. Sequential technique was carried out when primary antibodies were raised in similar species, whilst the parallel technique was carried out when primary antibodies were raised in different species. TLR1 and TLR4 were stained in the muscle sections along with markers for neutrophils (CD34), macrophages (CD68) and endothelial cells (CD31) to observe the distribution of TLR 1 and 4 in the muscle biopsies.

Similar to biotinylated immunohistochemistry, the human skeletal muscle sections were deparaffinised and antigens retrieved with citrate buffer. To block the non-specific binding of the antibodies, the slides were incubated in 1% BSA in PBS / 0.1% Tween 20 for 30 minutes at room temperature. TLR4, CD34, CD68 and CD31 antibodies were raised in mouse. Hence, the sequential technique was used to examine the co-distribution of these antigens in the same samples. TLR1 antibody was raised in rabbit, therefore the parallel technique was used to examine the co-distribution of TLR1 and TLR4.

For the sequential technique [Figure 13], slides were blocked with 1% BSA in PBS / 0.1% Tween 20 for 30 minutes at room temperature. This was followed by incubation of the slides with the primary antibody or their IgG isotype control in 1% BSA in PBS / 0.1% Tween 20 for 1 hour at room temperature, and then further incubation with the first secondary antibody, labelled with flurochrome-1, in 1% BSA in PBS / 0.1% Tween 20 for 1 hour at room temperature. After further wash with PBS, the second blocking step was performed, and then the second primary antibody and the second secondary antibody, labelled with fluorochrome-2, were applied in a similar manner to the first step. The slides were washed with PBS and coverslips were mounted using VECTASHIELD® mounting medium containing DAPI.

For the parallel technique [Figure 13], slides were incubated with the mixture of primary antibodies or their IgG isotype control in 1% BSA in PBS / 0.1% Tween 20 for 1 hour at room temperature. After washing with PBS, the slides were incubated with a mixture of the two secondary antibodies that were raised in different species with two different fluorochromes in 1% BSA in PBS / 0.1% Tween 20 for 1 hour at room temperature. The slides were washed with PBS and coverslips were mounted using VECTASHIELD® mounting medium containing DAPI. The slides were stored in the dark at -20 °C.

Cleaved Caspase-3 staining was also performed on paraffin embedded ischaemic mouse skeletal muscle sections to assess ischaemia-induced apoptosis in muscle. Similarly, the sections were deparaffinised and antigens were retrieved using citrate buffer. Sections were blocked with 1% BSA in PBS / 0.1% Tween 20 for 30 minutes at room temperature. After incubation with the primary antibody (Cleaved Caspase-3; 1:300; rabbit antibody; 9661; Cell Signalling) or its IgG isotype control (rabbit IgG) for 1 hour at room temperature, the slides were washed with PBS and treated with the secondary antibody (Alexa Fluor® 594 Chicken Anti-Rabbit IgG; 1:200; A-21442; Life Technologies). The slides were washed with PBS and coverslips were mounted using VECTASHIELD® mounting medium containing DAPI.

The images were viewed using a fluorescence microscope (Axioscop 2; Carl Zeiss Micro-imaging).

2.3.3. Immunocytochemistry

Immunocytochemistry was used to visualize the presence of desmin (marker for human skeletal muscle cell) in cultured human myotubes *in vitro*. There are two major types of immunofluorescence staining methods: 1) direct immunofluorescence staining in which the primary antibody is labelled with fluorescence dye, and 2) indirect immunofluorescence staining in which a secondary antibody labelled with fluorochrome is used to recognize a primary antibody.

The lists of the used primary and secondary antibodies are shown in Table 6 and Table 7.

Primary antibodies	Species	Suppliers	Concentrations
TLR4	Mouse	ab22048; Abcam	1:100
CD34	Mouse	ab9088; Abcam	1:100
CD68	Mouse	M0814; DakoCytomation	1:100
CD31	Mouse	M0823; DakoCytomation	1:20
TLR1	Rabbit	ab37068; Abcam	1:100

Table 6 – List of the primary antibodies

Secondary antibodies	Species	Suppliers	Concentrations
Alexa Fluor® 488 Anti-Rabbit IgG	Goat	A-11008; Life Technologies	1:200
Alexa Fluor™ 594 Anti-Rabbit IgG	Goat	A-11012; Life Technologies	1:200
Alexa Fluor® 594 Anti-Mouse IgG	Chicken	A-11032; Life Technologies	1:200
Alexa Fluor® 488 Anti-Mouse IgG	Chicken	A-1101; Life Technologies	1:200

Table 7 – List of the secondary antibodies

The indirect immunofluorescence staining was carried out on cultured human myotubes fixed on 8 chamber culture slides [Figure 14]. After fixation in ice-cold acetone for 10 minutes, the slides were placed in a humidifying chamber, washed with PBS for 5 minutes, and blocked with 10% chicken serum. Then, the slides were incubated in rabbit anti-human desmin antibody

(1:200; ab8592; abcam). After incubation with the primary antibody or its IgG isotype control (rabbit IgG) for 1 hour at room temperature, the slides were washed with PBS and treated for 30 minutes with the secondary antibody (Alexa Fluor® 594 Chicken Anti-Rabbit IgG; 1:200; A-21442; Life Technologies). The slides were washed with PBS and coverslips were mounted using VECTASHIELD® mounting medium containing DAPI. The slides were allowed to dry in darkness. Images were captured as soon as possible as the fluorescence is unstable and will become less fluorescent after a few days. The images were viewed using Axiotop 2 Mot plus microscope with Axiovision software and MRm camera (Carl Zeiss, Gottingen, Germany).

2.3.4. Haematoxylin & Eosin staining

Haematoxylin & Eosin (H&E) staining has been used for over a century and is still a recognised technique to observe the histomorphological changes in tissue sections. The stain displays a wide range of cytoplasmic, nuclear, and extracellular matrix features. Haematoxylin has a deep blue colour and acts as a basic dye, while Eosin is pink in colour and acts as an acid dye. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining (134). The Eosin staining will allow the non-nuclear tissue components to be clearly differentiated from each other.

In order to observe the histomorphological changes in gastrocnemius muscle in both human and mouse muscle biopsies, H&E staining was carried out on paraffin embedded gastrocnemius muscle sections. The ischaemic sections were compared with non-ischaemic ones to observe muscle fibre degeneration / regeneration, inflammatory cell infiltration, fibrotic tissue / adipose tissue replacement and necrosis.

The slides were placed in a rack and washed with xylene and ethanol to deparaffinise and rehydrate respectively. After washing the slides in distilled

water for 5 minutes, they were stained in Harris's Haematoxylin (Surgipath Europe Ltd, Peterborough, UK) for 6 minutes and washed again in running tap water for 20 minutes. The slides were decolorized in 1% acid alcohol for 1 second and washed in tap water for another 5 minutes. Further treatment with Lithium Carbonate for 3 seconds was performed, before washing in tap water and counterstaining in 1% Eosin for 15 seconds. Dehydration in 100% alcohol and xylene was carried out, before mounting sections with cover slips using DPX resin (BDH Guir®). Slides were examined under a light microscope and pictures taken with Zeiss Axiotop 2 Mot.

2.4. Human skeletal muscle cell culture

2.4.1. Myoblast isolation

Human gastrocnemius muscle was collected in solution A (3.6gr HEPES, 3.8gr NaCl, 0.112gr KCl, 47gr Glycerin, 500ml dH2O, PH=7.6) at the time of biopsy and stored at 4°C for no longer than 24 hours before isolation of human myoblasts.

The samples were transferred to weighing boats in a class II microbiological safety cabinet and cut into pieces of < $1mm^3$. Muscle fragments, mixed with solution ATE (4.5ml Trypsin-EDTA in 40.5ml solution A), were aspirated into flasks which then were placed in a beaker with water warmed to 37°C on a magnetic stirrer for 15 minutes to facilitate myoblast isolation.

The muscle fragments were allowed to settle at the bottom of the flask and only the remaining solution was transferred to 50ml tubes containing 20ml in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, Scotland) supplemented with 20% foetal calf serum, penicillin (100 U/ml), amphotericin B (25 µg/ml), streptomycin (100 µg/ml, all from Gibco). The 50ml tube was centrifuged at 13,000 rpm for 5 minutes, before discarding the medium and re-suspending the pellet in 1ml Skeletal Muscle Cell Growth Medium (SMM, Foetal Calf Serum, Fetuin, Epidermal Growth Factor, Basic Fibroblast Growth

Factor, Insulin and Dexamethasone; 23060; PromoCell, Germany) supplemented with 20% foetal calf serum, penicillin (100 U/ml), amphotericin B (25 µg/ml), streptomycin (100 µg/ml) (all from Gibco). The muscle fragments were re-suspended in solution ATE and the above steps were repeated to isolate more myoblasts in total volume of 3ml (135).

The cells were strained through the 40-micrometre cell strainer into a fresh 50ml tube, and were then plated into T12.5cm flasks and incubated at 37°C with 5% CO2. By the first 48 hours, extra 1ml of 20% SMM was added to the flasks. After a further 48 hours, the flasks were washed with PBS to remove erythrocytes and any remaining fragments while the myoblasts adherent to the flask were retained. The cells were then provided with fresh 4ml 20% SMM every 48 hours. In case of any contamination with endothelial cells, the culture medium was changed to a mixture of 20% DMEM and 20% SMM (50:50) for 48 hours, and then 20% DMEM only. The culture medium was switched back to 20% SMM when no endothelial cells could be visualized under the microscope.

Within 2 weeks, when cells were approaching 80% confluence, the flasks were treated with trypsin for 2 minutes at 37°C to detach the cells, which then were counted and plated at approximately 300,000 cells per T175 flask, 120,000 per T75 flask or 40,000 per T25 flask.

2.4.2. Myoblast differentiation, cell counting and freezing

Cells were inspected microscopically every 2-3 days to rule out any possible infection, and also to change the culture medium. At 80% confluence, cells were trypsinized: culture media was drained and the cells were washed with 2ml Trypsin-EDTA to neutralise the residual medium. Then, the cells were exposed to 5ml Trypsin-EDTA for 2 minutes at 37°C with mild agitation to help detach the cells. The contents of the flasks were subsequently transferred to a prefilled 50ml centrifuge tube with 20ml of 20% SMM to neutralize the trypsin-EDTA. After centrifuging the tubes at 13,000 rpm for 5

minutes, the supernatants were discarded and the cell pellets were re-suspended in 10ml of 20% SMM. These myoblast suspensions were diluted to be re-seeded in either another flask or a 6-well plate to proceed with differentiation. Myoblasts, which have undergone more than 10 passages, were not used in experiments.

In order to culture the myoblasts to myotubes, the myoblasts were plated onto 6 well culture plates (BD Falcon™, cat. no. 351143) or 8-well chamber slides (Falcon) and cultured in 20% SMM. At 80% confluence, cells were exposed to differentiation medium containing DMEM with 2% horse serum, penicillin (100U/ml), streptomycin (100 µg/ml) and amphotericin B (25 µg/ml) (all from Gibco). Within 7-10 days, differentiation was confirmed by observing the morphological changes under the light microscope at 20X magnification. Myotubes were elongated, thickened and multinucleated compared to the myoblasts (136). Immunohistochemical staining for desmin was also carried out to verify the myogenic phenotype of extracted myoblasts, with reported specificity and sensitivity of 99% and 71% respectively (137, 138).

A haemocytometer was used for cell counting before re-seeding the cells onto the flasks or plates. The haemocytometer is a device that can be used to count viable cells within a sample [Figure 15]. By knowing the dimensions (depth and area) of each segment of the haemocytometer, it is possible to calculate the concentration of cells within a known volume and therefore to calculate the concentration of cells in the fluid overall. Trypan blue, used to selectively colour dead cells that have damaged membranes, is added to the cell suspension to calculate the viability count.

After mixing the cell suspension with trypan blue (100 µl; 50:50), the haemocytometer was filled carefully with the mixture of cells and trypan blue, allowing capillary action to draw the solution out of the pipette. Only live cells were counted as oppose to those that had taken up the trypan blue.

Total cell count = ((4 corners of 16 squares) / 4) X 2 (dilution factor 1:2) = Number of cell X 10^4 per millilitre

For the purpose of long-term storage of the myoblasts, cells were trypsinized as described above, and centrifuged at 13,000 rpm for 5 minutes to produce a pellet. The supernatant was discarded and the pellet re-suspended in the 18ml of freezing medium (15ml 10% DMEM, 1.5ml Foetal Calf Serum and 1.5ml of DMSO). The resulting suspension was transferred to cryotubes in 1ml aliquots that were placed in a suitable freezing container and cooled down to -80°C for 24 hours, before being transferred to liquid nitrogen storage. In order to re-use the cells, the cryotubes were thawed quickly in a warm water bath and washed in culture medium to neutralize the effect of DMSO which is toxic to the cells. After centrifuging the suspension, the pellets were re-suspended in fresh culture medium and the cells were seeded into the flasks accordingly.

2.4.3. Simulated ischaemia

In order to investigate the functional consequences of ischaemia in cultured human myotubes, a model of simulated ischaemia was adopted from a previously published *in vitro* myotube ischaemia model from our lab (139) [Figure 16]. This reproducible model of myotube ischaemia mimics the critical parameters that occur in skeletal muscle ischaemia, including changes in the level of pH, LDH, pO_2 and CO_2. Culturing myotubes in depleted media, in a gas mixture containing 20% CO_2 and 80% N_2 for 6–12 h increases the pCO_2 and decreases the pH and pO_2 of culture media to similar levels that are observed in ischaemic skeletal muscle *in vivo*.

The cultured human myotubes in 6-well plates were placed in the sealed hypoxic chamber (Modular Incubator Chamber, MIC-101; Billups-Rothenberg, Del Mar, CA, USA) that was connected to a gas cylinder containing 20% CO_2 and 80% N_2. The chamber was flushed with the gas (10 litre per minute) for 20 minutes to minimize the amount of O_2 left in the chamber, before the gas was disconnected and the chamber was sealed completely [Figure 17]. Then, the chamber was placed in the incubator for 8

hours at 37°C. After microscopic examination of the plates to exclude floating cells, the plates were transferred immediately for sample collection for experiments. The media was collected in 20ml tubes, labelled and stored at -20°C for ELISA analysis. Cell lysates were prepared as described earlier for western blotting and co-immunoprecipitation.

2.4.4. Protein inhibitors

To investigate the TLR4 related downstream signalling pathway in simulated ischaemia, protein inhibitors were used to manipulate the signalling pathway at the level of both adaptor and transcription proteins *in vitro.*

The following inhibitors were added 6 hours prior to exposure to simulated ischaemia to inhibit the adaptor proteins: MyD88 inhibitor (20μM; tlrl-pimyd; Invivogen) and TRIF inhibitor (20μM; tlrl-pitrif; Invivogen). The following inhibitors were used to inhibit the transcription proteins: NF-kB inhibitor (10μM; Celastrol ant-cls; Invivogen) was added 6 hours prior to exposure to simulated ischaemia; P38 inhibitor (10μM; SB203580; SelleckChem, Houston, USA) was added 4 hours prior to exposure to simulated ischaemia; JNK inhibitor (20μM; 420119; Merck Chemicals Ltd, Nottingham, UK) was added 4 hours prior to exposure to simulated ischaemia. The different doses and the duration of the pre-treatment for the various inhibitors prior to the simulated ischaemia were adopted from the datasheets (IC50) provided by the manufacturers.

2.4.5. TLR4 antagonism

To investigate the functional consequences of TLR4 inhibition in ischaemic human myotubes, TLR4 was inhibited prior to simulated ischaemia *in vitro*.

The following inhibitors were used to inhibit the TLR4 receptor: Anti-human TLR4 neutralizing antibody (10 μg/ml; mabg-htlr4; Invivogen) was added 2 hours prior to exposure to simulated ischaemia and the ultrapure lipopolysaccharide from Rhodobacter sphaeroides (LPS-RS ultrapure TLR

antagonist) (10 μg/ml; tlrl-prslps; Invivogen) was added 4 hours prior to exposure to simulated ischaemia. The LPS-RS was also used for the *in vivo* experiments to inhibit TLR4 prior to the hind-limb ischaemia.

As a positive control, a synthetic TLR4 ligand was used: Synthetic Monophosphoryl Lipid A – MPLSs (50μM; tlrl-pimyd; Invivogen) was added 6 hours prior to exposure to simulated ischaemia.

2.5. Co-immunoprecipitation

Immunoprecipitation is a technique that enables the purification of a protein. The specific antibody against the protein of interest is incubated with the mixture of the proteins, and the antigen-antibody complex formed is then pulled out of the sample using protein A/G-coupled agarose beads. This method helps to physically isolate the protein of interest from the rest of the sample. Co-immunoprecipitation (Co-IP) is a similar technique that helps to identify physiologically relevant protein–protein interactions by targeting protein A with its relevant antibody to indirectly capture protein B which is bound to protein A. These protein complexes can be analysed to study the possibility of heterodimerisation of the proteins of interest.

2.5.1. Preparation of cell lysates

Co-IP technique was carried out to investigate the possibility of TLR4 and TLR1 heterodimerisation. Ischaemic and control human myotubes were lysed with RIPA buffer (25 mM Tris HCL pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Termo Scientific) as described earlier. As soon as the lysis process starts, proteolysis, dephosphorylation and denaturation happen. Keeping the samples on ice at all times and adding fresh protease and phosphatase inhibitors to the lysis buffer, slow down these events. Cell lysates (100μl) were incubated with either anti-TLR4 (2μg;

ab22048; abcam) or anti-TLR1 (2µg; ab37068; abcam) at 4^{o}C for 1 hour under gentle agitation.

Meanwhile, protein A-coupled sepharose beads (P9424; Sigma-Aldrich, UK) were prepared by incubating 100mg of beads in 1ml 0.1M PBS, then centrifuging and discarding the supernatant. After further incubation with 0.1% BSA in 1ml PBS for 1 hour and the subsequent wash with PBS and removal of the supernatant, lysis buffer (400µl RIPA) with added protease inhibitors was added to the slurry.

The slurry was mixed well and incubated with the samples (50µl of the beads per sample) for 2 hours at 4^{o}C under gentle agitation. Bound proteins were then washed twice with lysis buffer and the supernatant was removed after centrifugation. Following the removal of the supernatant after the second centrifugation, loading buffer (50µl per sample; Laemlli Blue) was added. Finally, the samples were boiled at 95^{o}C for 5 minutes to denature the proteins and separate them from the beads. After centrifuging the samples, the supernatants (containing the proteins) were collected. The immune complexes were then separated on SDS-PAGE gel, and immunoblotted (as described earlier) with TLR4 or TLR1 accordingly. Proteins were tagged with chemiluminescence, before the exposure to photographic film.

2.6. ELISA

Enzyme Linked ImmunoSorbent Assay (ELISA), introduced by Engvall and Pearmann in 1971 (140), is a biochemical technique that is used mainly to detect an antigen or an antibody in a mixture of proteins. This quantitative method provides a useful measurement of antigen or antibody concentration. There are two main variations in this technique: an ELISA can be used to detect an antigen that is recognised by an antibody (sandwich ELISA), or it can be used to test for an antibody that recognises an antigen.

The principles of the sandwich ELISA are as follows: the ELISA plate is incubated with the antibody of interest over a certain amount of time, and then the plate wells are washed and blocked with a blocking solution to minimize the non-specific binding. The diluted samples and standards are added to the wells and the plate is incubated until the antigen-antibody complex is formed. After incubating with the secondary antibody that is conjugated with Alkaline Phosphatase (AP) or Horseradish Peroxidase (HRP), the plate wells are washed and the substrate is added to produce light at a certain wavelength. Finally, the plate is placed into an ELISA microplate reader to determine the absorbance based on the wavelength. Knowing the concentration of the standards, the absorbance ratios of the samples are then calculated to determine the actual concentration [Figure 18].

2.6.1. Preparation of supernatants

As described earlier, at the time of preparing the cell lysates, the cell culture supernatants of the control and ischaemic cultured human myotubes were collected, labelled and stored at -20°C.

2.6.2. Preparation of blood samples

Whole blood (mouse model of hind-limb ischaemia; chapter 5) was collected in anti-coagulant (Sodium Citrate) containing syringes by puncturing the left ventricle at the time of sacrificing the animals. After centrifuging the samples at 3,000 rpm for 10 minutes, the supernatant (plasma) was collected and stored at -80°C.

The sandwich ELISA technique was carried out to detect the presence and to quantify the concentration of inflammatory cytokines (IL6, TNFα and INFα) and endogenous ligands (HSP60 and HSP70) in the *in vitro* experiments and inflammatory cytokines (IL6 and TNFα) in the *in vivo* experiments. The

following ELISA kits were used and the experiments were carried out as per manufacturer's instructions: Human VeriKine^{TM} INFα (41100; PBL Assay Science, USA), Human total HSP60 (1800-2; R&D Systems, USA), Human IL6 (D6050; R&D Systems), Human TNFα (DTA00C; R&D Systems), Human / Mouse total HSP70 (DYC1663-2; R&D Systems), Mouse IL6 (M6000B; R&D Systems) and Mouse TNFα (MTA00B; R&D Systems).

2.7. Polymerase chain reaction

Polymerase Chain Reaction (PCR) is an important technique that allows thermo-enzymatic quantification of DNA, based on the ability of DNA polymerase to synthesize new strands of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it is possible to delineate a specific region of template sequence to be amplified. This is facilitated by the use of primers, also known as qPCR probes, which are short pieces of single stranded DNA complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

Reverse transcriptase PCR analysis of mRNA, referred as RT-PCR, is one of many variants of PCR and is commonly used to detect RNA expression levels (141). Total or specific types of RNA are extracted from the samples, and then the RNA is reversed transcribed into single strand DNA, also known as complementary DNA or cDNA. After adding the primers to the transcribed cDNA, the PCR takes place and the polymerase carries out the amplification. Primers can be labelled with a wide range of fluorochrome tags. The use of fluorescence detection offers the advantage of sensitivity and a wide linear dynamic range for quantification. As the cycle number increases, the detected fluorescence also increases.

In order to measure TLR4 gene (507bp) expression *in vivo*, the mouse gastrocnemius muscle samples (mouse model of hind-limb ischaemia;

chapter 5) were collected in RNAlater at the time of sacrificing the animals at days 1, 3 and 21 post operation (hind-limb ischaemia / sham) and stored at -80^{o} C.

2.7.1. RNA extraction and quantification

RNA was extracted using the RNAeasy mini kit (74106; Qiagen, Germany) and a tissue lyser (Qiagen) [Figure 19]. A maximum amount of 20mg of RNAlater stabilized tissue was processed from each sample as per capacity of RNeasy spin column and the lysing capacity of the Buffer RLT. The lysis buffer (310µl per tube; 10µl 2-Mercaptoethanol in 1ml of RLT) and the samples were added into 2ml microcentrifuge tubes containing one stainless steel bead. Tubes were placed in the TissueLyser Adapter Set 2 x 24, and the TissueLyser was run for 2 x 3 minutes at 20Hz. Tubes were rearranged half way through to allow even homogenisation. The homogenate was centrifuged at 12,000 rpm for 10 minutes at 4^{o}C to remove insoluble material. Supernatant was transferred to a new tube and then left at room temperature for 5 minutes to promote dissociation of nucleoprotein complexes. After adding 70% ethanol (1:1 (v/v)), the total liquid was transferred to an RNeasy spin column placed in a 2ml collection tube and centrifuged at 10,000 rpm for 15 seconds. Further washing and centrifuging were carried out with RW1 and RPE buffers respectively. The RNeasy spin column was placed in a new 1.5ml collection tube and 50µl of RNeasy-free water was added directly to the spin column membrane.

The quantity of the total RNA was measured using a Thermo Scientific NanoDrop ND-1000 spectrophotometer (220nm-750nm). Each sample was pipetted (1.5 µl) onto the end of a fibre optic cable in the lower pedestal that was brought into contact with the second fibre optic cable, resulting in the liquid to bridge the gap between the fibre optic ends. A flash lamp provided the light source and a spectrometer was used to analyse the amount of ultraviolet irradiation absorbed by the nucleic acid. The NanoDrop spectrophotometer was calibrated using a diethylpyrocarbonate-treated water

blank. Measurements were taken at 260nm and the quantity of extracted RNA was calculated in ng/µL. Samples were then stored at -80^{o} C.

2.7.2. Reverse transcription and quantitative PCR

QuantiTect Reverse Transcription Kit (205313; Qiagen) was used to perform the reverse transcription. After thawing the samples on ice, the genomic DNA elimination reaction (2µl of gDNA wipeout buffer and 1µg of template RNA in RNase-free water; total volume: 14µl) was carried out for 2 minutes at 42^{o}C. Then, the template RNA (14µl) was added to tubes containing reverse transcription master mix (1µl of Quantiscript Reverse Transcriptase, 1µl of RT Primer Mix and 4µl of Quantiscript RT Buffer; total volume: 6µl) and incubated for 15 minutes at 42^{o}C. After further incubation for 3 minutes at 95^{o}C, the samples were ready for real-time quantitative PCR, also known as qPCR.

SensiMix SYBR HiROX Kit (QT605-02; Bioline Reagents Ltd, London, UK) and the 5' sense and 3' antisense TLR4 primers, provided with positive control double stranded DNA (rtp-mtlr4; Invivogen, USA), were used to carry out the real-time PCR.

In order to normalize the data, it is required to measure an endogenous unregulated reference gene, also called a housekeeping gene. This measurement enables the calculation of the fold expression in each sample, whereas the expression of the target gene is adjusted with its corresponding housekeeping gene that acts as a loading control. In this experiment, mouse tubulin (350bp), Mm-Tubb4a (251664; Qiagen) was used.

The PCR cocktails (10µl of Sensi Mix, 2µl of TLR4 or Tubulin primers, 4µl of template (cDNA (100 ng per reaction), negative control (PCR water) or positive control) and 4µl of PCR water; total volume: 20µl) were placed into the Corbett Rotor-Gene 6000 (Qiagen) [Figure 20] that was programmed for 40 cycles at 95^{o}C for 15 seconds, 60^{o}C for 15 seconds and 72^{o}C for 15 seconds. The data were recorded and the fold expression of the TLR4 was

calculated using ΔΔCt technique as described below. Further, the PCR products were separated on 2% agarose gel and visualized under UV light [Figure 20], to help better understanding of the data.

2.7.3. ΔΔCt method and melting peaks

Relative expression of the real-time PCR products was determined by using the ΔΔCt method to calculate the fold expression of the target gene that is normalized with the housekeeping gene. The positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold background level. Ct levels are inversely proportional to the amount of target nucleic acid in the sample, i.e., the lower the Ct level the greater the amount of target nucleic acid in the sample. The ΔCt was calculated by subtracting the Ct Tubulin from Ct TLR4 in either treatment or no-treatment group. The ΔΔCt was then calculated by subtracting the ΔCt (no-treatment group) from ΔCt (treatment group). The fold expression was calculated as $2^{-\Delta\Delta ct}$ ($\Delta Ct = Ct_{Tubulin} - Ct_{TLR4}$; $\Delta\Delta Ct = \Delta Ct_{treatment\ group} - \Delta Ct_{no\text{-}treatment\ group}$; Fold Expression = $2^{-\Delta\Delta ct}$).

Further, to check that the signal measured was from the cDNA of interest, all PCR products for the particular primer pair were expected to have the same melting temperature. Therefore, after real-time PCR amplification, a melting curve was performed. The temperature was raised by a fraction of a degree and the change in fluorescence was measured. At the melting peak temperature (Tm), fluorescence rapidly decreases as the two strands of DNA separate.

2.8. Mouse model of hind-limb ischaemia

In order to study the therapeutic potential of modulating TLR4 signalling in ischaemic skeletal muscle, the mouse model of hind-limb ischaemia was utilised. This is described in detail in chapter 5.

2.8.1. Haemodynamics and Laser Doppler Imaging

Laser Doppler Perfusion Imaging (LDPI) is a non-invasive technique facilitating the monitoring of tissue perfusion and haemodynamics (142). Laser Doppler measures the total local perfusion within capillaries, arterioles, venules and shunting vessels.

The depth of the measurement depends on the structure and density of the capillary bed, pigmentation and oxygenation. Further parameters that affect the depth of the measurement are the wavelength of the laser light and the distance between the sending and receiving fibres in the laser Doppler probe. The sending fibre scatters the light waves into the tissue that partly absorbs the energy, whilst some light waves are reflected back and detected by the returning fibre, converted into an electronic signal and analysed. The light hitting moving blood cells undergoes a change in wavelength (Doppler shift), while light hitting static objects is unchanged. The magnitude and frequency distribution of these changes in wavelength are directly related to the number and velocity of the blood cells in the sample volume.

In order to monitor tissue perfusion *in vivo* (mouse model of hind-limb ischaemia; chapter 5), the anaesthetized mice were positioned under the scanner head (Moor FLPI; Moor Instruments Ltd, Devon, UK) on a low temperature heating pad to maintain normal body temperature of 37°C, with 30cm distance between the animal and the scan head. The mice were placed in supine position and serial color-coded perfusion images were taken at days 1, 3 and 21 post operation (hind-limb ischaemia / sham). The images were then analysed using Moor FLPI review V3.0 software [Figure 21].

2.8.2. Blood oxygen pressure and preparation of blood samples

Underlying acid-base disturbances are inevitable in tissue ischaemia. Arterial blood gas analysis reveals oxygenation status, adequacy of ventilation and acid-base balance.

Arterial blood samples are used for blood gas analysis to study the parameters that can potentially be confounding factors secondary to ischaemia, including pH, pO_2, pCO_2, Hb, SO_2, Lactate, Glucose, Sodium and Potassium.

Whole blood (mouse model of hind-limb ischaemia; chapter 5) was collected in anti-coagulant (Sodium Citrate) containing syringes by puncturing the left ventricle at the time of sacrificing the animals. Air bubbles were removed and the needles were sealed with a rubber stopper to prevent the influx of air. The samples were kept in ice and transferred to the lab within 30 minutes for the analysis (RAPIDPoint 500 Systems; Siemens, Germany).

2.9. Power calculations

For human tissue analyses, sample size calculations were performed using the formula: $N = 2 \times (Z\alpha/2 + Z\beta)^2 \times \sigma^2/\delta^2$ (N represents the sample size per group; Zα/2 and Zβ represents the standard normal deviates for type I and type II errors; σ^2 represents the squared standard deviation; and δ^2 represents the squared differences between the treatment and control groups). From preliminary Western blot results from the human muscle biopsies, $\delta = 0.550$ and $\sigma = 0.321$, therefore to provide an 80% power of detecting a difference between the 2 groups ($Z\beta=0.84$) at 5% significance level ($Z\alpha/2=1.96$), N per group was calculated to be 6.07.

For *in vivo* studies, 6 animals of each strain were used for each time point. This number was chosen based on published studies of TLR changes in ischaemia as well as studies employing similar analyses in mouse hind-limb ischaemia models (48, 143).

2.10. Statistical analysis

JMP® 11.0.0 software (SAS institute, NC, USA) was used to present, describe and analyse the data from this study.

The Chi square test was carried out to compare the demographics between the patients with CLI and patients with no PAD. In human tissue experiments, the data (not normally distributed) was described as medians and ranges and compared with the Mann Whitney test. Presenting the *in vitro* experiments, the data (not normally distributed) was described as medians and ranges and compared with the Kruskal Wallis test. Presenting the *in vivo* experiments, the data (both normally and not normally distributed) was described as medians and ranges and compared with the ANOVA test.

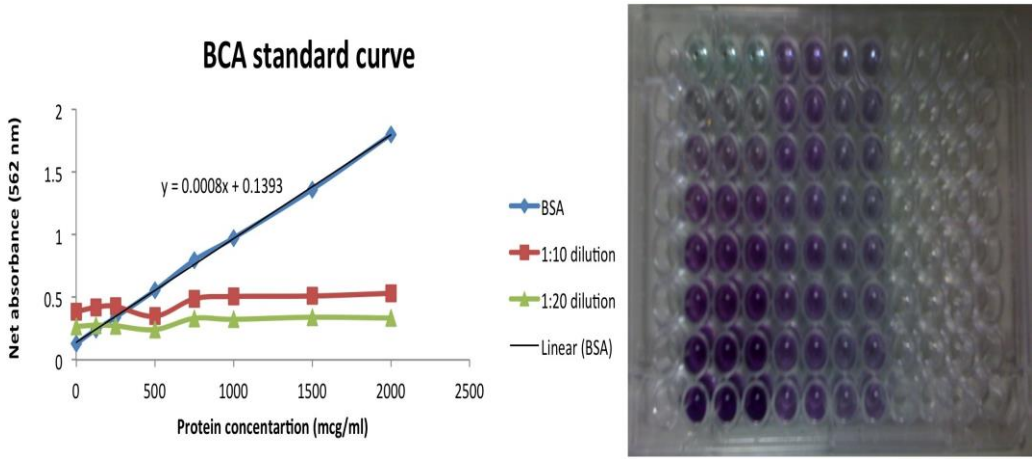


Figure 10 - The graph shows the standard curve (blue line) which is used to calculate the protein concentration in the samples (red and green lines), as per light absorbance. The picture shows the macroscopic colour differences between the standards (left 3 columns) and the samples (right 4 columns)

Figure 11 - Photograph of XCell Sure Lock electrophoresis devices

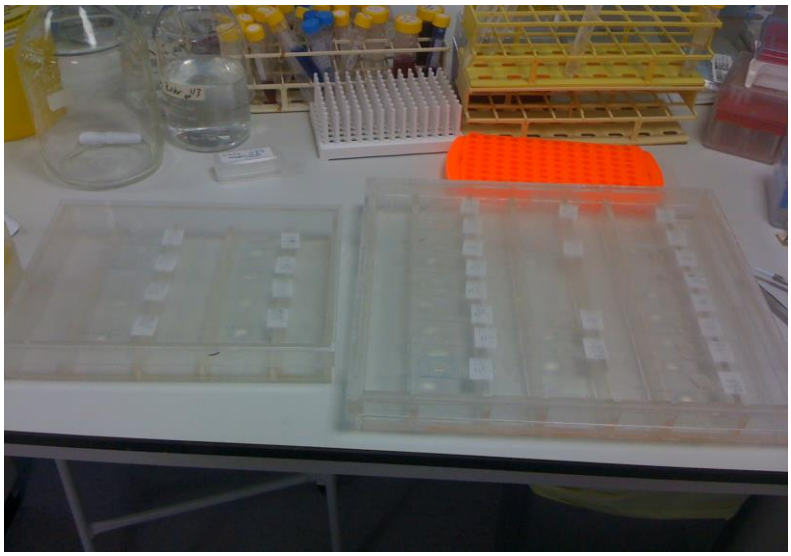


Figure 12 - Photograph of slides placed in a humidified chamber prior to staining

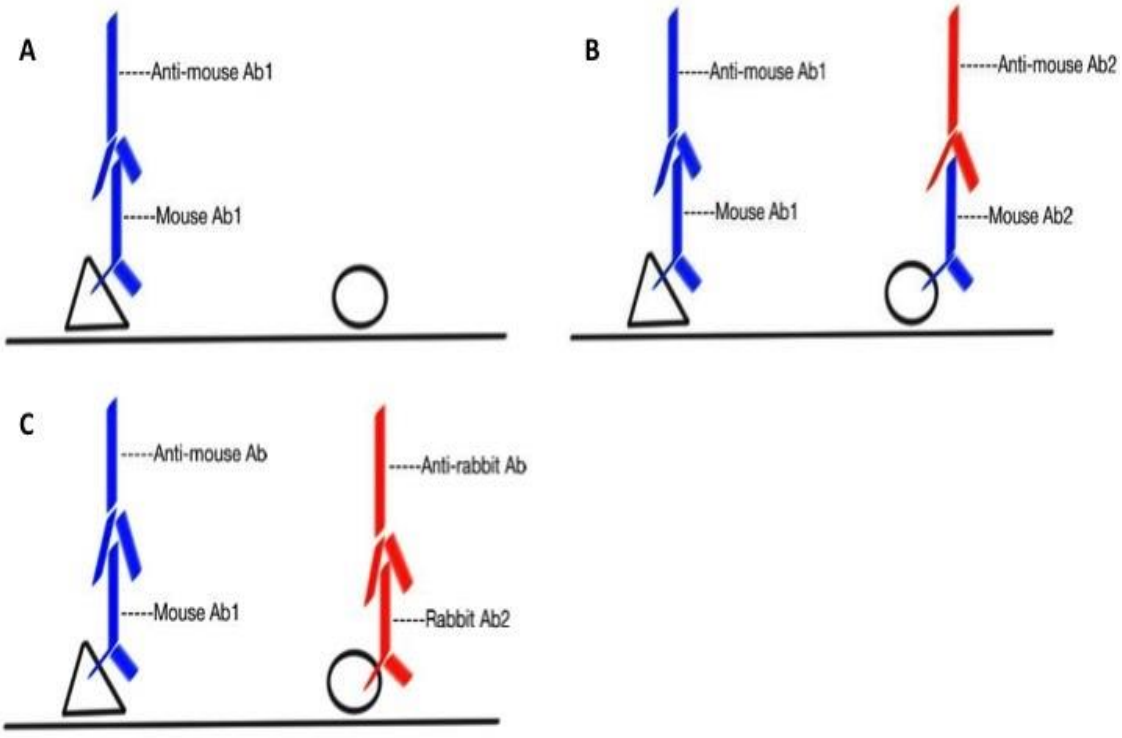


Figure 13 - A) Mouse-mouse sequential double staining in blue and red - step1; B) Mouse-mouse sequential double staining in red and blue - step2; C) Mouse-rabbit parallel double staining in red and blue

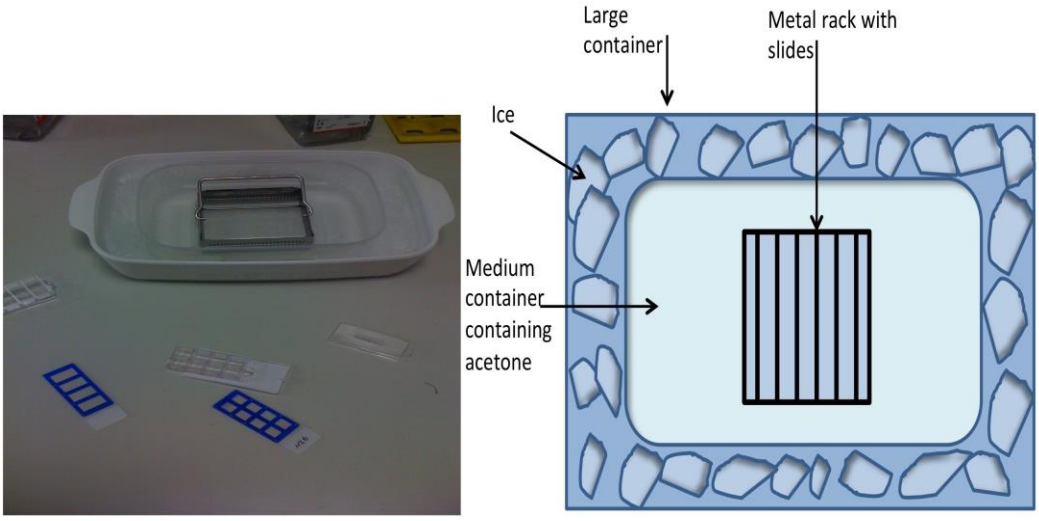


Figure 14 - Picture of the 8 chamber culture slides that were used to culture human myotubes prior to staining for desmin

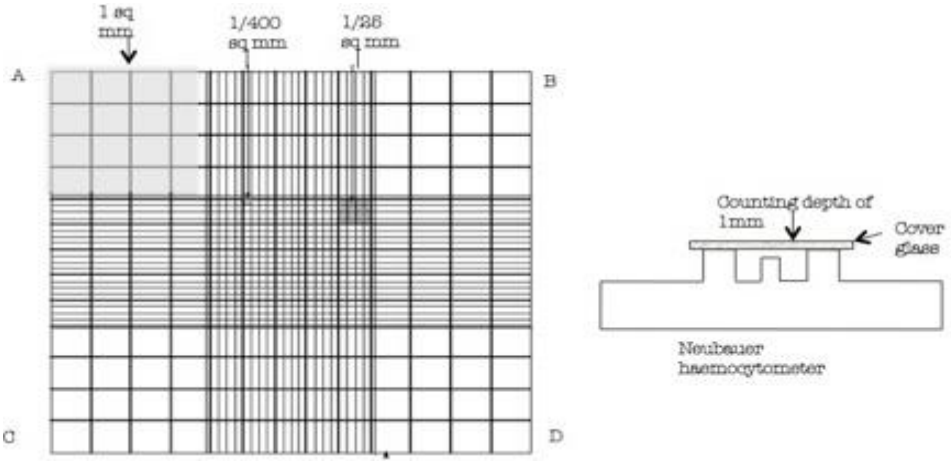


Figure 15 - Picture illustrates the haemocytometer device used for the purpose of cell counting

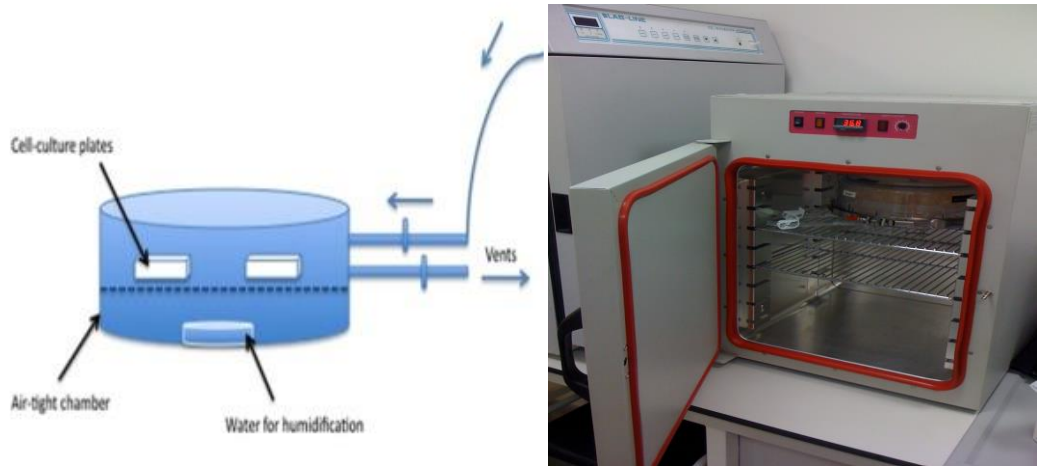


Figure 16 - This picture illustrates the hypoxic chamber used to induce myotube ischaemia *in vitro*. The chamber was incubated for 8 hours at 37°C

Figure 17 - Photographs showing cultured human myotubes placed in the hypoxic chamber connected to the gas cylinder for 20 minutes, before incubation for 8 hours

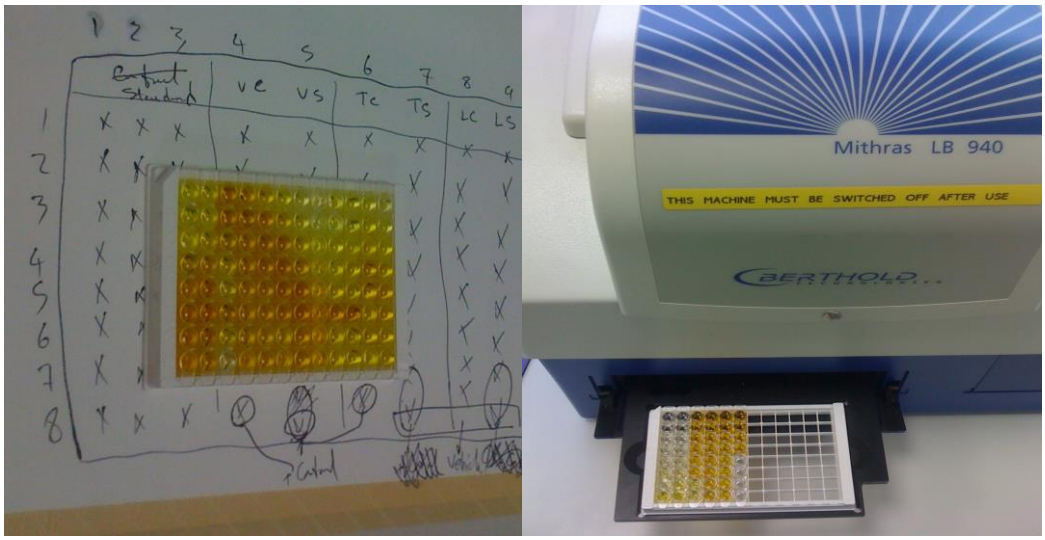
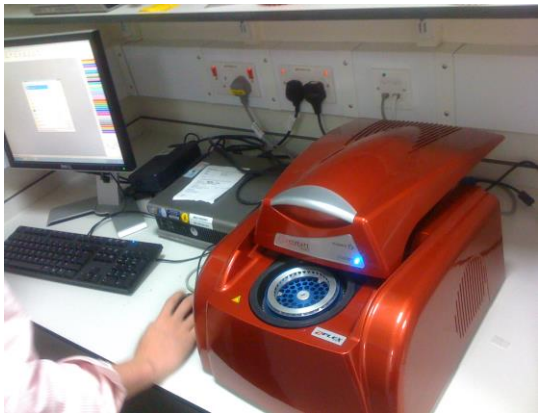


Figure 18 - The pictures show the ELISA plate after adding the substrate and the plate reader

Figure 19 - The picture shows the tissue lyser used for the extraction of the total RNA from the mouse skeletal muscle



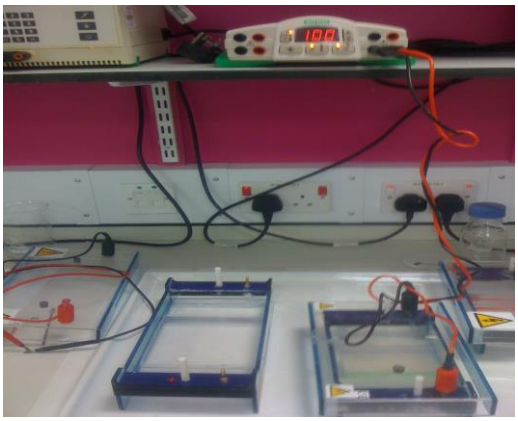


Figure 20 - The pictures show the Rotor-Gene 6000, separation of the PCR products on 2% agarose gel and the visualization under UV light

Figure 21 – The pictures show the Laser Doppler Perfusion Imager and the positioning of the mouse at the time of imaging

CHAPTER 3: EXPRESSION, DISTRIBUTION AND POTENTIAL ACTIVATION OF TLR4 AND TLR1 PATHWAY IN CRITICALLY ISCHAEMIC HUMAN SKELETAL MUSCLE

3. 1 Introduction

3. 2 Aims

3. 3 Experimental design

3. 4 Results

3. 5 Discussion

3. 1 Introduction

There has been a common misunderstanding that CLI simply represents the natural progression of IC in patients with PAD. Haemodynamics and blood flow are not the only contributing factors in the clinical presentation of PAD (144). Lower limb skeletal muscle is an important end organ in the pathophysiology of PAD. Ischaemia-induced skeletal muscle damage manifests as IC as well as poor functional performance in CLI.

Skeletal muscle forms the bulk of the lower limbs and play a key part in its function. Further, skeletal muscle has a role as a paracrine signalling organ and potential source of DAMPs which are host biomolecules that can initiate and augment a non-infectious inflammatory response. Normal physiology of the skeletal muscle consists of a stable tissue with very little turnover of nuclei, however in the event of any injury, the skeletal muscle has the ability to initiate the repair process. The initial phase of muscle repair is characterised by necrosis of the damaged tissue and activation of the inflammatory response (145).

The critical consequence of skeletal muscle ischaemia is an inadequate oxygen delivery or availability at the cellular level. Skeletal muscle function can be further compromised if exercise takes place during the hypoxic cycle. However, in comparison with other tissues such as brain and heart, skeletal muscle is relatively tolerant to hypoxia, probably due to the energy buffering system. Chronic hypoxia results in reduced fibre area and muscle mass to improve oxygen diffusion into muscle cells (146). Further, hypoxia is a trigger of oxidative stress due to the increase in the generation of reactive oxygen species (ROS) (147).

Oxidative stress-induced injury and inflammation are important considerations in skeletal muscle damage in patients with CLI. Critical reduction in blood flow in the lower limb, not only promotes generation of ROS, but also activation of leukocytes resulting in inflammation (148). Mitochondria is the main source of

ROS secondary to leakage of electrons. The impairment of the mitochondrial respiratory chain increases the leakage of electrons that results in an increase in ROS. Decrease in mitochondrial density and impairment of its activity associated with increased ROS production, have been observed in hypoxic human skeletal muscle (149-151). The ROS released during hypoxia act as signalling agents that trigger diverse functional responses, including activation of gene expression through the stabilization of the transcription factor hypoxia-inducible factor (HIF-α) (151). ROS can lead to cell damage and homeostatic disruption in skeletal muscle, and cause damage through lipid oxidation and modification of protein and DNA (152, 153).

In patients with IC, during exercise induced ischaemia of the lower limbs, increased anaerobic metabolism can lead to alteration of pH, muscle cell permeability and ultimately result in muscle damage. During the rest period, restoration of the blood flow in the ischemic muscle activates inflammation. Further, the pro-inflammatory cytokines released from the damaged muscle may trigger the systemic inflammatory response.

The immune system is divided into innate and acquired immunity. The innate immune system is primarily a prophylactic system, where toll-like receptors (TLR) play a key role in regulating the inflammatory response. The role of ROS in the activation of the innate immune system via TLR4 and its NFk-B signalling pathway has already been shown (154-156). ROS does not directly activate TLR4, but causes several DAMPs which can activate TLR4, resulting in the release of pro-inflammatory cytokines. This is a process of sterile inflammation.

TLR4 can recognise specific ligands via its three-dimensional structure. The ability to recognise and bind with more ligands is further enhanced by the help of co-receptors such as CD14 and MD2, and the ability of the TLRs to form heterodimers. Upon recognition of a ligand by TLR4, the adaptor proteins, MyD88 and TRIF, are activated with further downstream signalling

to phosphorylate the transcription proteins such as NFk-B, JNK and P38, which then result in production of inflammatory cytokines.

TLR1, 2 and 6 form heterodimers with TLRs present in the cell membrane (38). TLR2 and TLR6 have been shown to form heterodimers with TLR4. It has been demonstrated that these heterodimers can initiate a sterile inflammatory response in brain tissue in response to endogenous ligands (157, 158). The assembly of these heterodimers is regulated by co-receptors such as CD36, while the downstream signalling pathway appears to be the same as when these receptors are activated individually (158). Although TLR1 is known to form heterodimers with other TLRs, it is unclear whether its assembly with TLR4 improves the ligand recognition or alters the response.

The role of TLR4-mediated inflammatory damage in chronic ischaemia has been shown in ischaemic myocardial dysfunction. TLR4, expressed by cardiomyocytes, mediates post infarct maladaptive left ventricular remodelling and impairs cardiac function after myocardial infarction probably via inflammatory cytokine production and matrix degradation (159). Further, silencing TLR4 in cardiomyocytes improved cell survival following hypoxic injury through reduced inflammatory and apoptotic signals (160). In another study, it was shown that cardiomyocytes can initiate the inflammatory cascade by means of expressing TLR4 that responds to both pathogen and damage associated molecular patterns. The binding of TLR4 with Heat Shock Protein 60 (HSP60) was also observed in chronic heart failure cardiomyocytes. Furthermore, HSP60 induced robust production of inflammatory cytokines in chronic heart failure cardiomyocytes, which was reduced by TLR4-blocking antibodies. The authors concluded that the expression, ligand-binding capacity and pro-inflammatory function of cardiomyocyte TLR4 are up-regulated after myocardial infarction, which promote inflammation and exacerbate heart failure (161).

Other studies have shown the expression and upregulation of TLR4 in human skeletal muscle in other pathologies such as obesity, type 2 diabetes and

chronic kidney disease (162-164). TLR4-mediated inflammation has been shown in a mouse model of skeletal muscle ischaemia-reperfusion (165), although the mechanism is not fully understood. Preliminary work from our group has shown that expression of TLR4 is upregulated in gastrocnemius muscle of patients with CLI (38).

3. 2 Aims

The aims of these experiments were to study in further detail the TLR4 pathway in skeletal muscle biopsies obtained from patients with CLI and those form patients without PAD, and to study the TLR4 distribution in critically ischaemic human skeletal muscle.

Objective:

The objectives were:

1) To define changes in TLR4 distribution and protein expression in human CLI tissue
2) To identify downstream signalling pathway and potential endogenous ligands involved

Hypothesis:

It was hypothesized that TLR4 is upregulated in critically ischaemic human skeletal muscle and is activated by endogenous ligands within the ischaemic tissue.

3. 3 Experimental design

Human skeletal muscle biopsies were taken from the medial head of gastrocnemius from patients undergoing major lower limb amputation for CLI (n=6) and from patients with no PAD undergoing saphenous vein harvesting for CABG surgery (n=6) [Table 8].

Parameters	CLI group (n=6)	Control group (n=6)	P value
Sex (male)	4	3	> 0.05
Age (mean, years)	61.5	64.5	> 0.05
HTN,	3	4	> 0.05
Diabetes	4	4	> 0.05
Smoking	3	2	> 0.05
eGFR (mean, mls/min/1.73m^2)	61	71	> 0.05
Hyperlipidaemia	5	4	> 0.05
Symptoms of PAD	6	0	**< 0.05***
ABPI < 0.9	6	0	**< 0.05***

Table 8 – Demographics of the 2 patient groups. The 2 groups differed only in terms of the presence of PAD symptoms and reduced ABPI, where p<0.05, Chi square test

Immunofluorescence was carried out to study TLR4 and TLR1 expression and distribution in human skeletal muscle biopsies including co-location with endothelial cells, neutrophils and macrophages [Figure 22].

Western blot analyses were carried out to study TLR expression and activation in human skeletal muscle biopsies. Further western blot analyses were carried out to identify the downstream signalling mechanisms contributing to cellular damage in ischaemic human skeletal muscle. The amount of transcription factors was quantified in the human skeletal biopsies utilising western blot analyses [Figure 23]. Western blot images were analysed by Image J software (Chapter 2.2) to measure the intensity of the bands of interest, adjusted by their corresponding tubulin loading controls.

The data were found to be not normally distributed, therefore the Mann-Whitney U test was used to compare differences between the 2 groups.

The primary antibodies used for the western blotting were as follows:

Antibodies	Suppliers	Concentrations
TLR1	ab37068; Abcam	1:1000
TRIF	ab13810; Abcam	1:1000
TLR4	ab22048; Abcam	1:500
HSP70	sc-373867; Santa Cruz	1:1000
HSP60	sc-136291; Santa Cruz	1:1000
MyD88	ab2064; Abcam	1:1000
P-P38	D3F9; Cell Signalling	1:1000
P38	9212; Cell Signalling	1:1000
JNK	9252; Cell Signalling	1:1000
P-JNK	9251; Cell Signalling	1:1000
NFkB	C22B4; Cell Signalling	1:1000
P-NFkB	93H1; Cell Signalling	1:2000
Cleaved Caspase-3	9661; Cell Signalling	1:1000
β tubulin loading	ab4074; Abcam	1:1000

The primary antibodies used for the Immunofluorescence were as follows:

Primary antibodies	Species	Suppliers	Concentrations
TLR4	Mouse	ab22048; Abcam	1:100
CD34	Mouse	ab9088; Abcam	1:100
CD68	Mouse	M0814; DakoCytomation	1:100
CD31	Mouse	M0823; DakoCytomation	1:20
TLR1	Rabbit	ab37068; Abcam	1:100

The secondary antibodies were as follows:

Secondary antibodies	Species	Suppliers	Concentrations
Alexa Fluor® 488 Anti-Rabbit IgG	Goat	A-11008; Life Technologies	1:200
Alexa Fluor™ 594 Anti-Rabbit IgG	Goat	A-11012; Life Technologies	1:200
Alexa Fluor® 594 Anti-Mouse IgG	Chicken	A-11032; Life Technologies	1:200
Alexa Fluor® 488 Anti-Mouse IgG	Chicken	A-1101; Life Technologies	1:200

3. 4 Results

The immunofluorescence staining demonstrated that both TLR4 and TLR1 are expressed on endothelium (CD31), neutrophils (CD43) and macrophages (CD68) in muscle biopsies from patients with CLI [Figure 24 (a, b)]. TLR4 and TLR1 double staining of ischaemic muscle sections showed co-localization of these receptors, suggestive of heterodimerisation [Figure 24 (a, b)].

To investigate further the potential of TLR4 and TLR1 heterodimerisation, more detailed analyses were carried out on cultured human myotubes

(Chapter 4). Western blot analyses of muscle homogenates showed upregulation of TLR4 (median 0.84, range 0.50-1.07) and TLR1 (median 2.34, range 1.86-3.77) in ischaemic samples *versus* non-ischaemic samples (TLR4, median 0.29, range 0.007-0.57, $P<0.05$, n=6; TLR1, median 1.23, range 0.022-2.72, $P<0.05$, n=6) [Figure 25]. Transcription factors in human muscle biopsies were quantified showing increased phosphorylated NFkB (median 0.41, range 0.25-0.85) and phosphorylated JNK (median 0.21, range 0.12-0.31) in ischaemic samples *versus* non-ischaemic samples (pNFkB, median 0.12, range 0.02-0.25; pJNK, median 0.02, range 0.009-0.05, $P<0.05$, n=4) [Figure 25]. More detailed analyses on the downstream signalling mechanism were carried out on cultured human myotubes (Chapter 4).

Western blot analyses of cleaved caspase 3 expression were performed to measure apoptosis within the muscle biopsies and this was found to be increased in ischaemic samples (median 0.15; range 0.059-0.387), *versus* non-ischaemic samples (median 0.001, range 0.0007-0.0187, $P<0.05$, n=4) [Figure 26]. Further, protein levels of HSP 60 and 70, potential endogenous ligands of TLR4, were found to be elevated in CLI biopsies (HSP60, median 0.106, range 0.082-0.119; HSP70, median 0.087, range 0.065-0.095) *versus* non-ischaemic biopsies (HSP60, median 0.041, range 0.028-0.044; HSP70, median 0.0305, range 0.023-0.038, $P<0.05$, n=4) [Figure 26].

3. 5 Discussion

From analyses of human tissue, it was demonstrated that muscle biopsies contained macrophages, neutrophils and endothelial cells in addition to muscle fibres. Immunofluorescence staining showed the presence of both TLR4 and TLR1 on neutrophils, endothelium and macrophages. The presence of TLR4 and TLR1 in these cells have already been shown in previous studies (166, 167). These findings confirm wide distribution of both TLR4 and TLR1 in the lower limb which may emphasise the role of these

receptors in the ischaemia-induced inflammatory response in patients with CLI. Double staining of the sections showed co-localisation of TLR4 and TLR1. The potential for heterodimerisation is investigated in more detail in *in vitro* experiments described in Chapter 4.

TLR4 and TLR1 were found to be present in muscle biopsies from both groups, with significant upregulation of these receptors in the ischaemic samples. The considerable change in TLR4 expression suggests that it plays an important role in critically ischaemic skeletal muscle. The upregulation and potential adverse effects of TLR4 in lower limb skeletal muscle have been shown in previous studies (168) where TLR4 was upregulated and activated by specific ligands to initiate an inflammatory process in skeletal muscle after seven consecutive days of bed rest in healthy older adults.

Phosphorylated NFkB and JNK, activated forms of the key transcription proteins (NFkB and JNK) in the TLR4 signalling pathway, were significantly upregulated in the ischaemic samples, indicating activation of these pathways and is suggestive of functional upregulation of TLR4 in critically ischaemic muscle. However further experiments are required to confirm these observations and to better understand the downstream signalling involved.

Joshi *et al*. showed that cleaved caspase 3 is upregulated in ischaemic muscle (139), indicating the activation of the apoptotic caspase 3 pathway. The response is dependent on the severity of the ischaemia, suggesting that multiple pathways play a role in triggering caspase 3 activation (169). TLR-induced inflammation and apoptosis has been shown to be associated with increased cleaved caspase 3 (170). The increase in cleaved caspase 3 in our experiments suggest that TLR4 upregulation and activation in critically ischaemic muscle may contribute to the increased apoptosis that occurs.

The upregulation of HSP60 and 70, potential TLR4 endogenous ligands, were shown in the ischaemic samples. Liu *et al*. showed that HSP70 is increased in ischaemic skeletal muscle in patients with chronic lower limb ischaemia while HSP70 expression varies with regard to clinical stages (171).

Our findings support the presence and release of potential DAMPs in ischaemic muscle which could contribute to chronic activation of TLR4 and the resulting tissue damage.

Limitations:

The control experiments were carried out on skeletal muscle biopsies obtained from patients undergoing CABG surgery. Although the patients in the control group had no history of arterial claudication, with normal readings of ABPI, there is possibility for some underlying PAD as these patients share most of the cardiovascular risk factors. Further, presence of potential infection in the biopsies is another limitation of these experiments, as infecting microorganisms are potent TLR4 activators. To reduce the risk of infection, the biopsies were taken away from macroscopically infected areas. Further, due to the limited amount of tissue biopsies available from each control patients, some experiments were carried out on only 4 patients. Also, we did not study specific contribution of TLR4 receptors on various cell types, but autocrine/paracrine effect of tissue as a whole. Finally, these experiments were observational only but important to study human tissue to understand relevance in patients, and next chapter will use in vitro model to investigate functional effects.

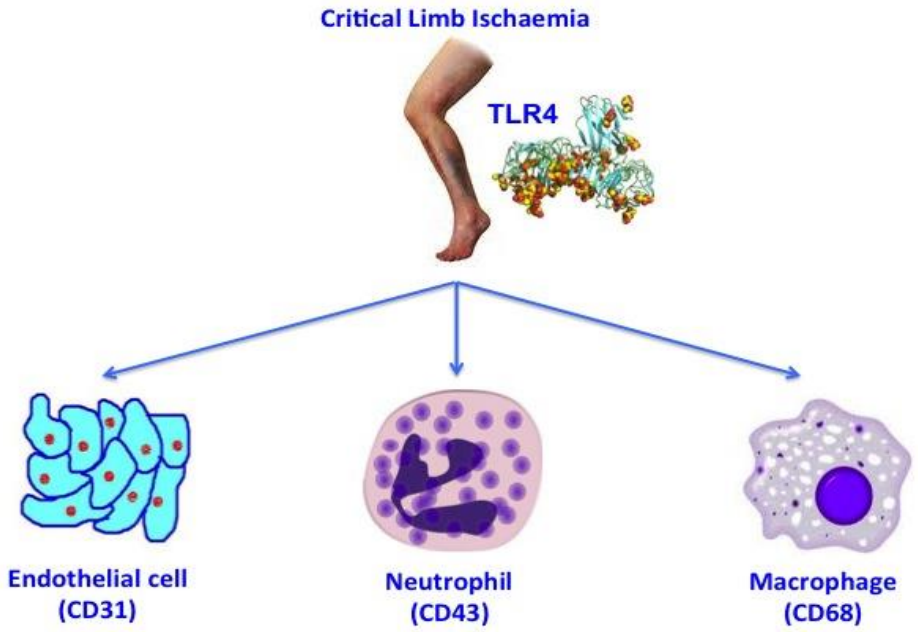


Figure 22 - CD31, CD43 and CD68 were stained as specific markers for endothelial cells, neutrophils and macrophages respectively

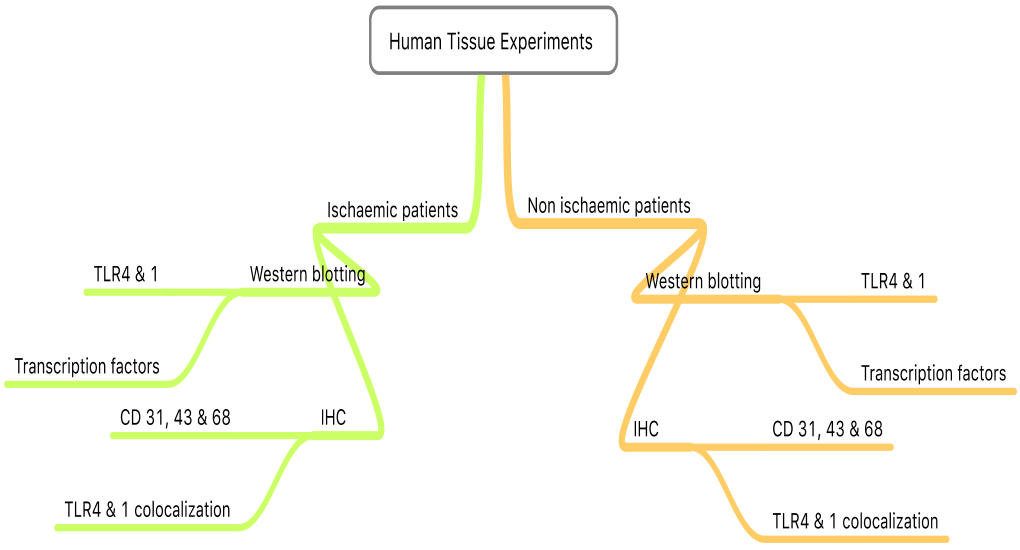


Figure 23 – Experimental design

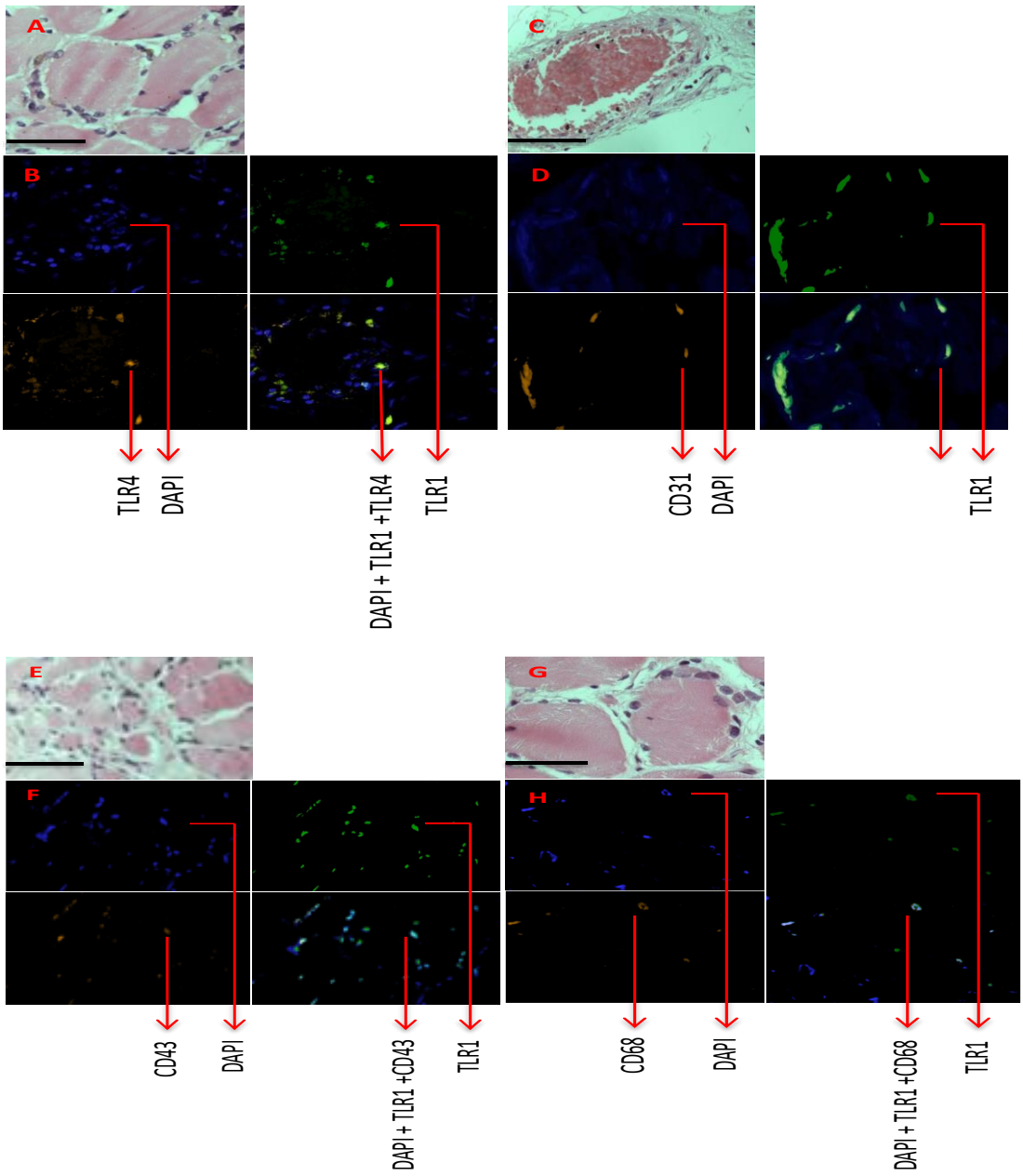


Figure 24 (a) - H&E and immunohistochemical staining of ischaemic muscle sections A) H&E staining of ischaemic muscle section B) Double labelled Fluor-IHC-P staining of TLR1 & TLR4 showing co-localisation of TLR1 & TLR4 C) H&E staining of micro vessel within ischaemic muscle D) Double labelled Fluor-IHC-P staining of TLR1 & CD31 showing TLR1 expression by endothelial cells E) H&E staining of ischaemic muscle section F) Double labelled Fluor-IHC-P staining of TLR1 & CD43 showing TLR1 expression by neutrophils G) H&E staining of ischaemic muscle section H) Double labelled Fluor-IHC-P of TLR1 & CD68 showing TLR1 expression by macrophages (A, C, G: scale bar = 10µm; E: scale bar = 50µm)

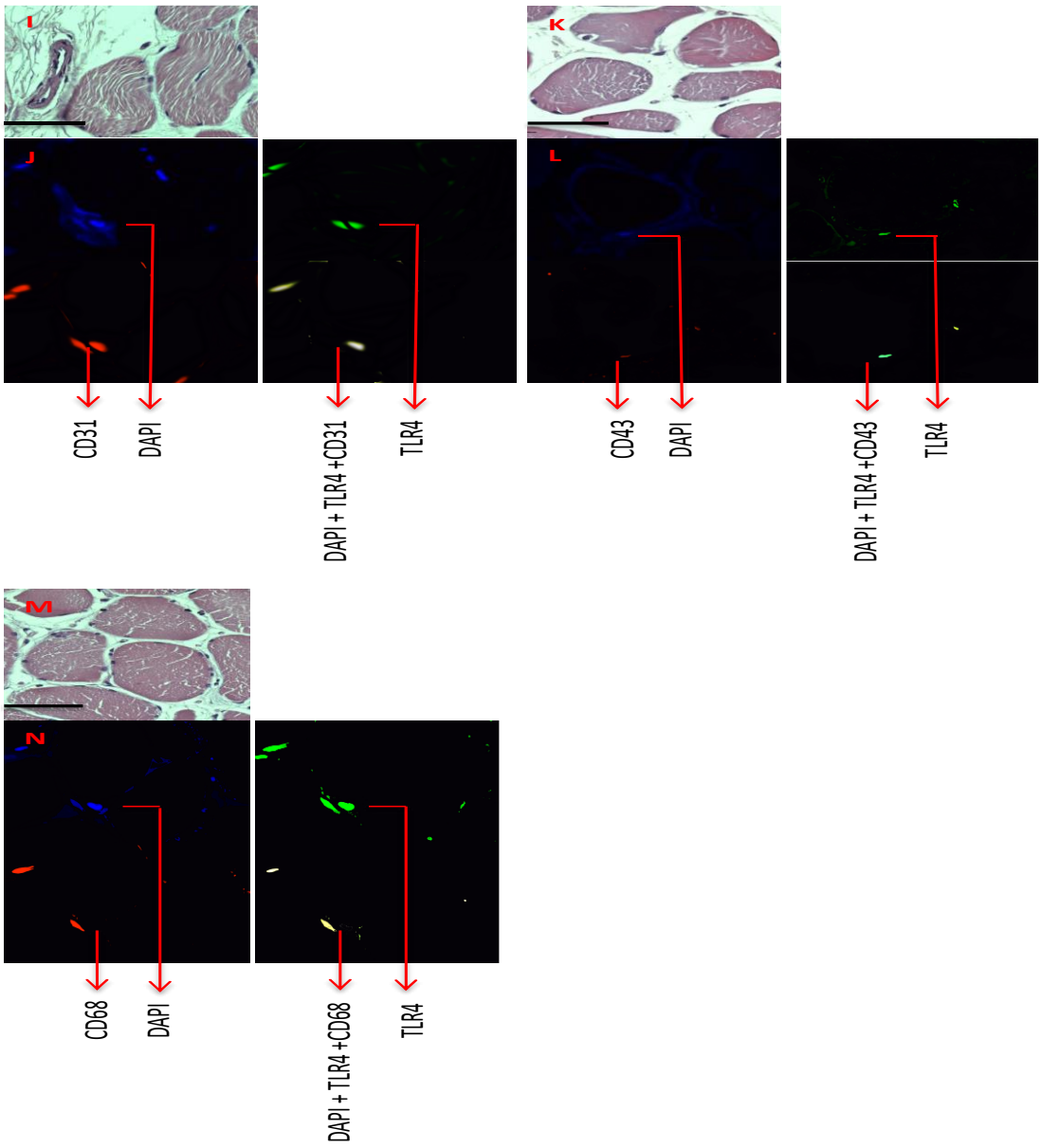


Figure 24 (b) - H&E and immunohistochemical staining of ischaemic muscle sections I) H&E staining of micro vessel within ischaemic muscle J) Double labelled Fluor-IHC-P staining of TLR4 & CD31 showing expression of TLR4 by endothelial cells K) H&E staining of ischaemic muscle section L) Double labelled Fluor-IHC-P staining of TLR4 & CD43 showing TLR4 expression by neutrophils M) H&E staining of ischaemic muscle section N) Double labelled Fluor-IHC-P staining of TLR4 & CD68 showing TLR4 expression by macrophages (I, K, M: scale bar = 10μm)

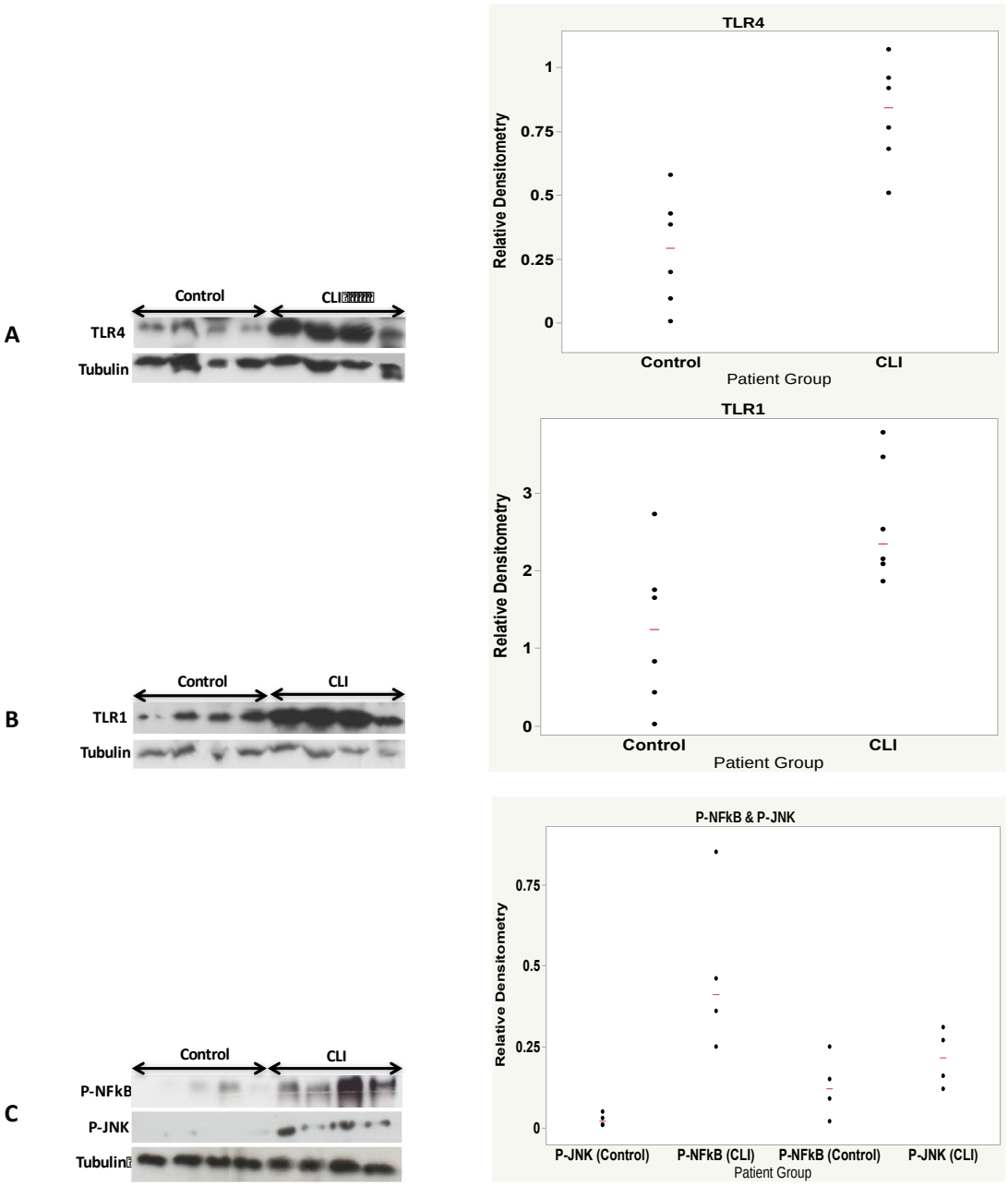


Figure 25 - A) Representative immunoblots for TLR4 and tubulin loading control and densitometric analysis of the TLR4 immunoblots (n=6, P<0.05) B) Representative immunoblots for TLR1 and tubulin loading control and densitometric analysis of the TLR1 immunoblots (n=6, P<0.05) C) Representative immunoblots for P-NFkB, P-JNK and tubulin loading control & densitometric analysis of the P-NFkB and P-JNK immunoblots (n=4, P<0.05)

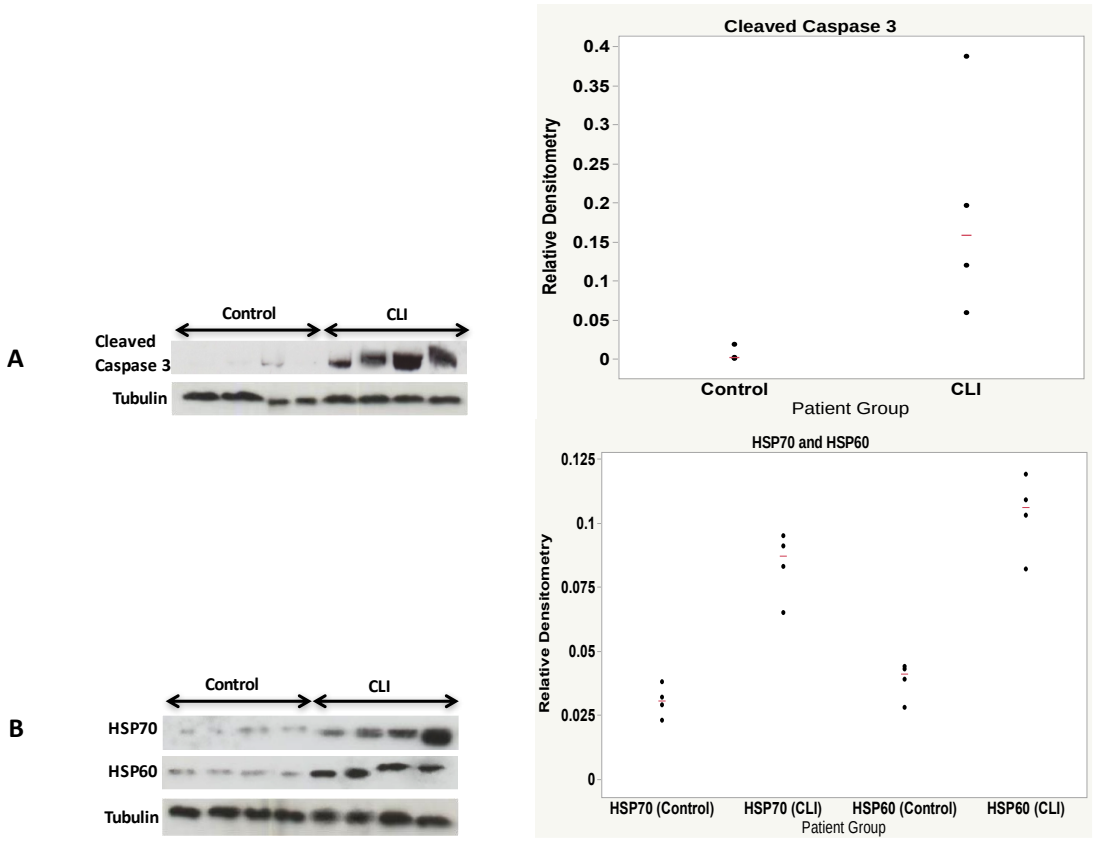


Figure 26 - A) Representative immunoblots for cleaved caspase 3 and tubulin loading control and densitometric analysis of the cleaved caspase 3 immunoblots (n=4, P<0.05) B) Representative immunoblots for HSP70, HSP60 and tubulin loading control & densitometric analysis of the HSP70 and HSP60 immunoblots (n=4, P<0.05)

CHAPTER 4: FUNCTIONAL CONSEQUENCES OF TLR4 ACTIVATION IN ISCHAEMIC HUMAN MYOTUBES

4. 1 Introduction

4. 2 Aims

4. 3 Experimental design

4. 4 Results

4. 5 Discussion

4. 1. Introduction

In vitro experiments are beneficial in enhancing our understanding of how a specific harmful effect may affect a living system. Further, these experiments allow us to study a single effect of an action in isolation and offer high sensitivity without interaction from other biological pathways. Moreover, *in vitro* models have the potential to generate reliable data to identify areas which can be further investigated in *in vivo* models enabling the reduction in the number of animals required and refinement of animal experiments.

Human skeletal muscle cell line is a valuable tool to study the pathophysiology of the skeletal muscle. Human myotubes have been utilized to study muscle physiology, aetiological investigations and therapeutic potentials in skeletal muscle related pathologies. Multiple human skeletal muscle culture systems have been developed which are mainly based on cell isolation from healthy donors or individuals with the relevant disease (172).

Skeletal muscle has a remarkable ability to regenerate after injury. In the event of injury, skeletal muscle undergoes degeneration and regeneration process. This regeneration process relies on the interactions between the satellite cells and their environment (173). Satellite cells, upon activation, can re-enter the cell cycle to proliferate and differentiate into myoblasts where further differentiation and proliferation result in formation of myocytes which then fuse to form multinucleated myotubes. Myoblasts can be isolated from the muscle sample by different approaches: enzymatic digestion or cell migration (174, 175). The in vitro culture of proliferating and differentiating myoblasts has been shown to be an effective method in the preparation of cell samples suitable for morphological and cytochemical analyses (136).

The isolated myoblasts are grown to confluence before differentiation induction. Activated proliferating myoblasts express MyoD as a myogenic marker (176). The full maturation of the human skeletal muscle cells is evident when myotubes exhibit striated band pattern. The striated patterns indicate the formation of organized sarcomeres, the basic contractile

apparatus of skeletal muscle cell, meaning that these myofibres are structurally capable of contraction (177).

Human skeletal muscle fibres express three types of myosin heavy chain (MyHC) including MyHC-β, MyHC-2A and MyHC-2X. The expression of the MyHC isoform defines the muscle fibre phenotype as slow fibres (type 1 muscle fibre) or fast fibres (type 2 muscle fibre). Most human muscles consist of both fibre types in different degrees based on their functional demand, age and gender (178).

It has been demonstrated that cultured myotubes retain the characteristics of their donor, as the donor phenotype is to some extent carried over to myotubes *in vitro*. The retention of the metabolic characteristics of the muscle cell donor is not fully understood, however a combination of genetic and epigenetic mechanisms are probably involved (179).

During muscle hypoxia, ATP synthesis is impaired and therefore a mismatch of ATP need to ATP production occurs, leading to decreased tissue ATP resources and damage to the ATP-dependent enzymes in the skeletal muscle cells. The disturbed oxygen homeostasis will result in cell oedema and unspecific activation of proteases (180), which results in cell death and inflammation which later is followed by repair and formation of new muscle fibres (181). In the presence of persistent ischaemia and tissue hypoxia, skeletal muscle responds by changes in gene expression. Hypoxia inducible factor 1 (HIF-1) coordinates the cellular response in an attempt to recapture oxygen homeostasis (182).

Ischaemia comprises of the accumulation of metabolic waste and nutrition depletion in addition to hypoxia. Exposure of cultured myotubes to nutrition depletion, hypoxia and hypercapnia is associated with increased HIF-1 levels, and simulates certain characteristics of skeletal muscle ischaemia (139). These characteristics are described as a fall in the pH of the culture media, morphological changes in myotubes and condensation of nuclear chromati,

increased level of cleaved caspase 3, and increased LDH release by myotubes (139).

The cultured human myotubes from patients with no PAD can provide a platform for *in vitro* experiments to assess the functional consequences of ischaemia when the cell lines are exposed to simulated ischaemia. Further comparison between the cultured myotubes from patients with no PAD and patients with CLI can demonstrate if cultured myotubes from ischaemic skeletal muscle retained any memory and behave differently.

TLR4 has been shown to be up-regulated and activated in cultured human endothelial cells when simulated ischaemia is introduced. This up-regulation has been associated with activation of the inflammatory system (183).

The presence of TLR4 in cultured human myotubes can be assessed in cell lines obtained from patients with CLI and patients with no CLI. The downstream signalling pathway can also be examined when the effect of protein inhibitors can be observed at different levels targeting TLR4, and its adaptor and transcription proteins. Further, release of endogenous ligands such as HSP 60 and 70 can be examined in the process of hypoxia-related skeletal muscle cell damage.

Intracellular and extracellular physiological cascades are policed by activation and inhibition of various biological markers. Natural or synthetic inhibitors have become the basis for the development of therapeutic agents. Protein inhibitors are useful tools for research and clinically, for the treatment of different medical conditions. Application of protein inhibitors in *in vitro* experiments has enabled scientists to investigate downstream signalling pathways more specifically.

TLR4 inhibitors have played a pivotal role in the investigation and understanding of the role of TLR4 in the pathophysiology of different diseases. The impact of TLR4 inhibitors on cardiovascular dysfunction have been described in the literature (42).

4. 2. Aims

The aims of these experiments were to study the role of the TLR4 signalling pathway in ischaemia-induced cellular damage and inflammation in human skeletal myotubes and to assess for any differential response between myotubes cultured from patients with CLI and those from patients without PAD.

Objectives:

The objectives were:

1) To isolate myoblasts from patients with CLI and from patients without PAD and to culture them to myotube stage
2) To compare specific baseline characteristics relating to the TLR4 pathway in both types of myotubes
3) To assess the response of these myotubes to ischaemia
4) To understand the role of TLR4 signalling pathway in ischaemia-induced apoptosis and cytokine release from myotubes
5) To explore the role of TLR4 / downstream signalling pathway inhibition in ischaemia-induced apoptosis and cytokine release from myotubes
6) To explore the possibility of heterodimerisation of TLR4 and TLR1

Hypothesis:

It was hypothesized that:

1) TLR4 is up-regulated and its signalling pathway activated in cultured human myotubes exposed to simulated ischaemia [Figure 27].
2) TLR4 inhibition attenuates ischaemia-induced damage in cultured human myotubes exposed to simulated ischaemia [Figure 28].
3) MyD88 downstream signalling pathway is involved in regulating inflammatory gene transcription in TLR4-induced skeletal muscle damage in ischaemia [Figure 29].

4) NFkB, JNK and P38, as transcription proteins, are involved in regulating inflammatory gene transcription in TLR4-induced skeletal muscle damage in ischaemia [Figure 30].
5) Simulated ischaemia induces inflammatory cytokine release from skeletal myotubes, via a mechanism that involves heat shock protein (HSP) 60&70, known endogenous ligands of TLR4 [Figure 31].
6) Simulated ischaemia results in heterodimerisation of TLR4 and TLR1 in human myotubes.

4. 3. Experimental design

The overall experimental design for the *in vitro* work is illustrated in Figure 32 and details of experimental techniques are given in Chapter 2. Human gastrocnemius muscle biopsies were taken from patients with CLI undergoing major lower limb amputation (Group 1; n=6) and from patients with no PAD (Group 2; n=6). Human myoblasts were isolated, cultured to myotubes and then exposed to simulated ischaemia. The isolated myoblasts were examined every 48 hours to monitor for confluence before induction to differentiate into myotubes [Figure 33]. Same numbers of cultured cells were placed in culture plates to normalise the amount of proteins in all the experiments. Further, fluorescent immunohistochemical staining for desmin, a marker of human skeletal muscle, was carried out to confirm the presence of myogenic cell lines [Figure 34].

Western blot analyses of TLR4, TLR1 and cleaved caspase 3 were carried out on the cell lysates to assess TLR4 expression, signalling activity and apoptosis respectively. Further, ELISA quantification of IL-6 and TNFα were carried out on supernatants to quantify the inflammatory cytokine response after exposure to simulated ischaemia [Figure 32].

Cultured human myotubes were pre-treated with 1) TLR4 agonist (Synthetic Monophosphoryl Lipid A), TLR4 neutralizing antibody or TLR4 antagonist (RS-LPS) prior to exposure to simulated ischaemia: Western blot analyses of

TLR4, P-NFkB (signal-related kinase) and cleaved caspase 3 were carried out on cell lysates to assess TLR4 expression, downstream signalling activity and apoptosis respectively; 2) MyD88 or TRIF inhibitor prior to exposure to simulated ischaemia: ELISA analyses were carried out to quantify IL6 and TNFα release, and Western blot was used to assess expression of cleaved caspase 3 as a marker of apoptosis; 3) NFkB, JNK or P38 inhibitor prior to exposure to simulated ischaemia: ELISA analyses were carried out to quantify IL6 and TNFα release, and Western blot was used to assess the expression of MyD88, TRIF, NFkB, JNK, P38 and cleaved caspase 3; and 4) TLR4 neutralizing antibody prior to exposure to simulated ischaemia: ELISA analysis was carried out to quantify HSP60, HSP70, IL6 and TNFα release in the supernatants; and Western blot was used to assess expression of HSP60, HSP70 and cleaved caspase-3 in the cell lysates. Co-immunoprecipitation was carried out to assess the potential heterodimerisation of TLR4 and TLR1.

The following inhibitors were used to inhibit adaptor and transcription proteins, and TLR4 receptor. All doses were calculated based on the previous similar studies or information provided on the datasheet (IC50):

Inhibitors	Suppliers	Pre-conditioning period
MyD88	20μM; tlrl-pimyd; Invivogen	6 hours
TRIF	20μM; tlrl-pitrif; Invivogen	6 hours
NFkB	10μM; Celastrolant-cls; Invivogen	6 hours
P38	10μM; SB203580; SelleckChem	4 hours
JNK	20μM; 420119; Merck Chemicals	4 hours
TLR4 Neutralizing Ab	10 μg/ml; mabg-htlr4; Invivogen	2 hours
TLR4 antagonist (LPS-RS)	10 μg/ml; tlrl-prslps; Invivogen	4 hours
TLR4 agonist (LPS)	50μM; tlrl-pimyd; Invivogen	6 hours

The following ELISA kits were used and the experiments were carried out as per manufacturer's instructions:

ELISA kits	Suppliers
Human IL6	D6050; R&D Systems
Human TNFα	DTA00C; R&D Systems
Human total HSP60	1800-2; R&D Systems
Human total HSP70	DYC1663-2; R&D Systems
Human INFα	41100; PBL Assay Science

4. 4. Results

4.4.1 Myotubes derived from patients without PAD

Western blot analyses of lysates of myotubes derived from patients with no PAD showed that TLR4 (median 44; range 26-53) and cleaved caspase 3 (median 27; range 18-33) were up-regulated after exposure to simulated ischaemia ($P<0.05$; Mann Whitney test) compared to non-ischaemic myotubes from the same patient group (TLR4, median 14, range 6-18; cleaved caspase 3, median 6, range 4-8) [Figure 35].

The ELISA assessment of the supernatants of cultured myotubes derived from patients with no PAD showed increased expression of IL-6 (pg/ml) (median 178, range 133-269) and TNFα (pg/ml) (median 199, range 174-295) after simulated ischaemia ($P<0.05$; Mann Whitney test) [Figure 35]. Further ELISA quantification of the supernatants of myotubes from patients with no PAD showed significant increase in both HSP60 and HSP70 (HSP60, median 1240, range 950-2200 vs median 100, range 50-1000; HSP70, median 5900, range 4500-7000 vs median 1900, range 800-3000), when exposed to simulated ischaemia ($P<0.05$; Mann Whitney test) [Figure 36].

4.4.2 Myotubes derived from patients with CLI

The cultured myotubes from patients with CLI showed similar findings in terms of up-regulation of TLR4 (median 73; range 55-91 vs median 35, range 23-52, P<0.05; Mann Whitney test) and cleaved caspase 3 (median 28; range 21-47 vs median 8.5, range 5-13, P<0.05; Mann Whitney test), and increased expression of IL-6 and TNFα after simulated ischaemia (IL-6; median 207, range 180-225 vs median 35, range 20-49; TNFα, median 393, range 330-425 vs median 14, range 8-22, P<0.05; Mann Whitney test) compared to non-ischaemic myotubes from the same patient group [Figure 37]. Western blot analyses of the cell lysates confirmed the presence of both HSP60 and HSP70, and demonstrated up-regulation of these endogenous ligands in patients with CLI, when exposed to simulated ischaemia (HSP60, median 15, range 13-16 vs median 5, range 2.5-7; HSP70, median 19, range 17-21 vs median 11, range 7-14) (P<0.05; Mann Whitney test) [Figure 36]. ELISA quantification of the supernatants showed significant increase in both HSP60 and HSP70 in patients with CLI (HSP60, median 3800, range 2700-4500 vs median 1500, range 700-2400; HSP70, median 7000, range 5700-8000 vs median 3200, range 2500-4800), when exposed to simulated ischaemia (P<0.05; Mann Whitney test) [Figure 36].

4.4.3 Baseline differences between both groups

Myotubes from patients with CLI expressed greater levels of TLR4 as compared to those from patients with no PAD after exposure to the simulated ischaemia. Further, western blot analysis of TLR1 in cell lysates showed up-regulation in both groups after exposure to the simulated ischaemia (P<0.05; Mann Whitney test) [Figure 35, Figure 37].

4.4.4 TLR4 inhibition in myotubes derived from patients with CLI

Pre-treatment of myotubes from patients with CLI using TLR4 antagonist (RS-LPS) prior to simulated ischaemia reduced the expression of P-NFkB (pg/ml)

(median 20, range 14-31) in ischaemic cell lysates as compared to ischaemic cell lysates with no pre-treatment (median 40, range 35-51) (P<0.05; Mann Whitney test) [Figure 38]. Pre-treatment of myotubes from patients with CLI using TLR4 neutralizing antibody or TLR4 antagonist (RS-LPS) prior to simulated ischaemia reduced the expression of cleaved caspase 3 (TLR4 neutralising Ab, median 100, range 74-141; TLR4 antagonist RS-LPS, median 128, range 100-160) in ischaemic cell lysates as compared to ischaemic cell lysates with no pre-treatment (median 302, range 259-371) (P<0.05; Mann Whitney test) [Figure 39]. Further, pre-treatment with TLR4 agonist (LPS) was associated with increased expression of P-NFkB (median 56, range 50-66 vs median 40, range 35-51) (P<0.05; Mann Whitney test) [Figure 38]. The amount of P-NFkB, activated form of NFkB, was normalised as a ratio to the total levels of NFkB. ELISA quantification of the supernatants showed significant decrease in expression of endogenous ligands HSP60 (median 2230, range 1400-2900 vs median 3700, range 2100-4500) and HSP70 (median 3070, range 2500-4250 vs median 7000, range 6250-8100), as well as inflammatory markers IL6 (median220, range 200-255 vs median 105, range 70-155) and TNFα (median 398, range 310-476 vs median 145, range 110-245), when the cells are pre-treated with TLR4 neutralizing Ab prior to the simulated ischaemia (P<0.05; Mann Whitney test) [Figure 40].

4.4.5 Inhibition of adaptor proteins in myotubes derived from patients with CLI

Western blot analyses of the cell lysates from patients with CLI confirmed the presence of the adaptor proteins MyD88 and TRIF and their expressions were independent to the exposure to simulated ischaemia in culture [Figure 41]. Pre-treatment of myotubes from patients with CLI with MyD88 inhibitor prior to simulated ischaemia resulted in decreased IL6 (pg/ml) (median 59, range 40-101 vs median 205, range 180-245) and TNFα (pg/ml) (median 97, range 78-175 vs median 391, range 360-480) release compared to from

myotubes with no pre-treatment before the simulated ischaemia [Figure 42] and down-regulation of cleaved caspase-3 (median 56, range 42-78 vs median 128, range 102-160) [Figure 43] ($P<0.05$; Mann Whitney test). However, myotubes pre-treated with TRIF inhibitor disclosed no significant changes in IL6 and TNFα release or cleaved caspase-3 expression ($P>0.05$; Mann Whitney test). Further, the inhibition of the adaptor proteins prior to simulated ischaemia was associated with reduced expression of HSP60 (median 1600, range 1000-2100 vs median 3800, range 3000-4200) and HSP70 (median 3800, range 2700-5000 vs median 7000, range 5400-7800) ($P<0.05$; Mann Whitney test) [Figure 44, Figure 45].

4.4.6 Inhibition of transcription proteins in myotubes derived from patients with CLI

Expression of the activated form of NFkB (median 11, range 8-15 vs median 3, range 2-12), JNK (median 13, range 10-18 vs median 4, range 2-13) and P38 (median 18, range 15-19 vs median 8, range 5-12) were significantly increased after exposure to simulated ischaemia ($P<0.05$; Mann Whitney test) [Figure 41]. Pre-treatment of myotubes with NFkB, JNK and P38 inhibitor prior to ischaemia resulted in decreased levels of IL6 (pg/ml) (median 55, range 40-96 vs median 207, range 180-250) and TNFα release (median 55, range 40-96 vs median 493, range 330-425), and down-regulation of cleaved caspase-3 (median 23, range 15-43 vs median 140, range 110-146) ($P<0.05$; Mann Whitney test) [Figure 46] [Figure 47].

Further, the inhibition of the transcription proteins prior to simulated ischaemia was associated with reduced expression of HSP60 (median 1500, range 1100-1900 vs median 3800, range 3000-4200) and HSP70 (median 4200, range 3400-4900 vs median 7000, range 5400-7800) ($P<0.05$; Mann Whitney test) [Figure 44, Figure 45].

4.4.6 TLR4 and TLR1 heterodimerisation

TLR4 and TLR1 co-immunoprecipitation showed heterodimerisation of these receptors in cultured human myotubes after exposure to simulated ischaemia [Figure 48].

4. 5. Discussion

In this chapter, functional consequences of TLR4 activation in ischaemic human myotubes were examined *in vitro*.

It was demonstrated that TLR4 is present in cell lines derived from both patients with CLI and patients without PAD. Myotubes from both groups showed up-regulation of TLR4 after exposure to simulated ischaemia with the response from the CLI group being more pronounced, suggesting that cultured myotubes from patients with CLI kept their phenotype. Therefore, further work was carried out on the cultured myotubes from patients with CLI.

Up-regulation of TLR4 after simulated ischaemia is associated with activation of its downstream signalling pathway. Both MyD88 and TRIF adaptor proteins were found in the cell lysates with no significant changes in their expression after simulated ischaemia, although the inhibition of MyD88 was associated with reduced inflammatory response as compared to inhibition of the TRIF pathway. This suggests that the TLR4 response to simulated ischaemia is mainly regulated via the MyD88 signalling pathway rather than the TRIF pathway.

Moreover, the activated transcription proteins (NFkB, JNK and P38) were found to be upregulated in the cultured myotubes after exposure to the simulated ischaemia, while the inhibition of these regulatory proteins were associated with reduced inflammatory response. This suggests that these transcription proteins regulate the TLR4-induced inflammatory gene transcription. Simulated ischaemia was associated with increased cytokine release (IL6 and TNFα) and apoptosis (C-Caspase 3), which was attenuated

by the inhibition of TLR4 prior to simulated ischaemia. Investigation of HSP60 and 70 as potential endogenous ligands for TLR4, showed that these proteins are also upregulated in the presence of ischaemia-induced TLR4 activation.

Increased cytokine release, apoptosis and expression of HSP60 and 70 and TLR4 occurred in ischaemic skeletal muscle *in vitro*. TLR4 antagonism was associated with reduced apoptosis and inflammatory cytokine release and down-regulation of HSP60 and 70 expressions. This suggests a potential pathway where TLR4 and its endogenous ligands contribute to a positive feedback loop to maintain a pro-inflammatory environment during ischaemia.

Hence, there is a potential link between HSP60 and 70-induced inflammatory cytokine production and TLR4 activation in ischaemic human myotubes.

Finally, heterodimerisation of TLR4 and 1 in cultured human myotubes was demonstrated after exposure to simulated ischaemia. Further experiments are required to investigate the potential downstream signalling pathway after activation of heterodimerisation between TLR4 and 1, and whether a different downstream signalling pathway is mobilized.

In this chapter, it was shown that the myoblasts were successfully isolated and cultured to myotubes from patients with CLI, and these were different from those derived from patients without PAD. This highlights the importance of considering relevant cell lines for the study of diseases and their treatments. The response of CLI myotubes to further ischaemic insult and the effect of TLR4 downstream inhibition in this response demonstrate the involvement of the TLR4 pathway and suggests that this pathway may be a potential therapeutic target.

<u>Limitations:</u>

The experiments were carried out on cells isolated from patients with CLI, where the process of isolation, culture and differentiation may have affected their phenotype. Further, simulated ischaemic conditions do not fully

represent the complex pathophysiological conditions present in *in vivo*. Hence, it is important to use an *in vivo* model to further our understanding.

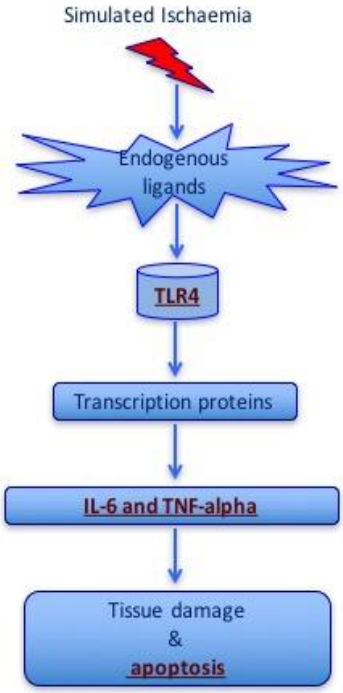


Figure 27 - TLR4 signalling pathway highlighting the potential up-regulation and activation of TLR4 in cultured human myotubes exposed to ischaemia

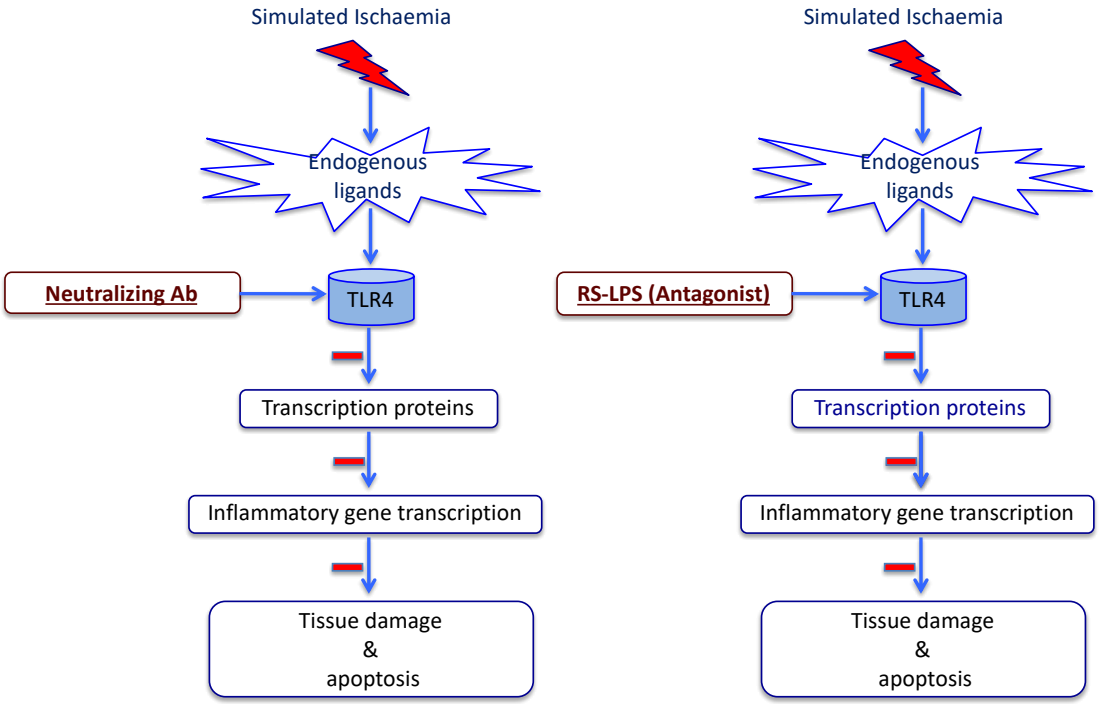


Figure 28 - TLR4 signalling pathway highlighting the potential effect of TLR4 inhibition (Neutralizing Ab or RS-LPS) on tissue damage in cultured human myotubes after exposure to ischaemia

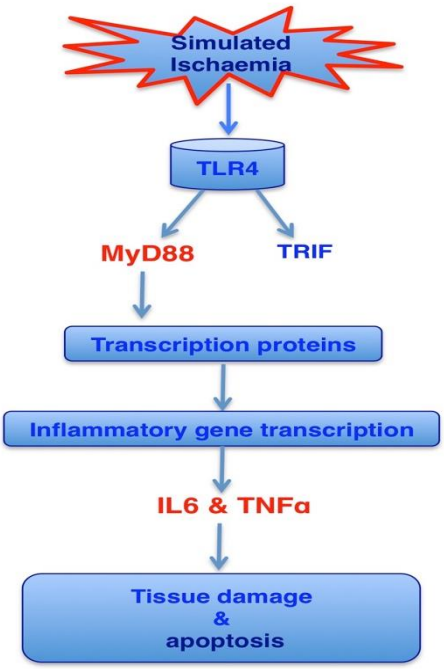


Figure 29 - TLR4 signalling pathway highlighting the potential role of MyD88 in downstream signalling pathway and the release of inflammatory cytokines

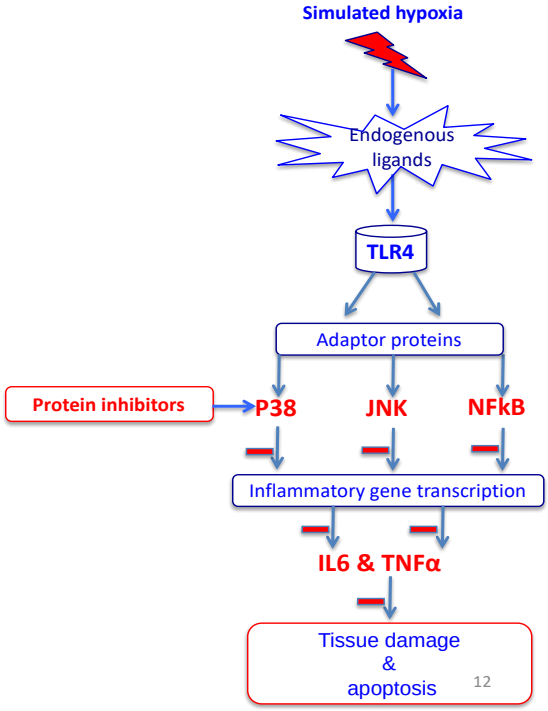


Figure 30 - TLR4 signalling pathway highlighting the potential roles of P38, JNK and NFkB, as transcription proteins, in the downstream signalling pathway and the release of inflammatory cytokines

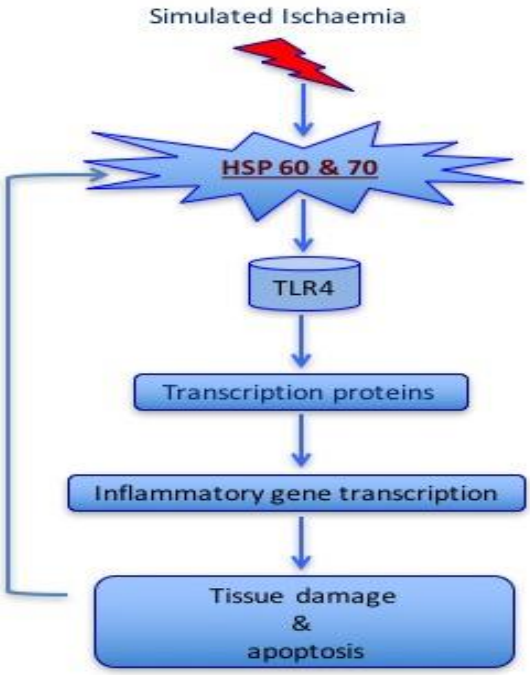


Figure 31 - TLR4 signalling pathway highlighting the potential role of HSP60 and HSP70, as endogenous ligands, activating the TLR4 pathway.

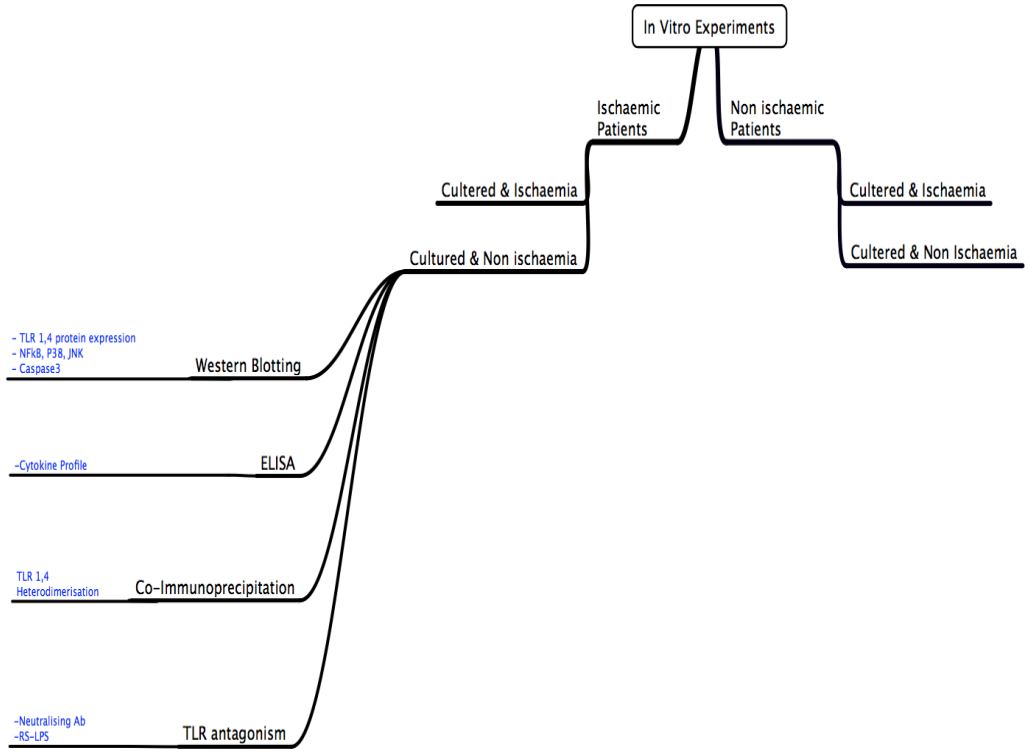


Figure 32 - Experimental design for the *in vitro* studies

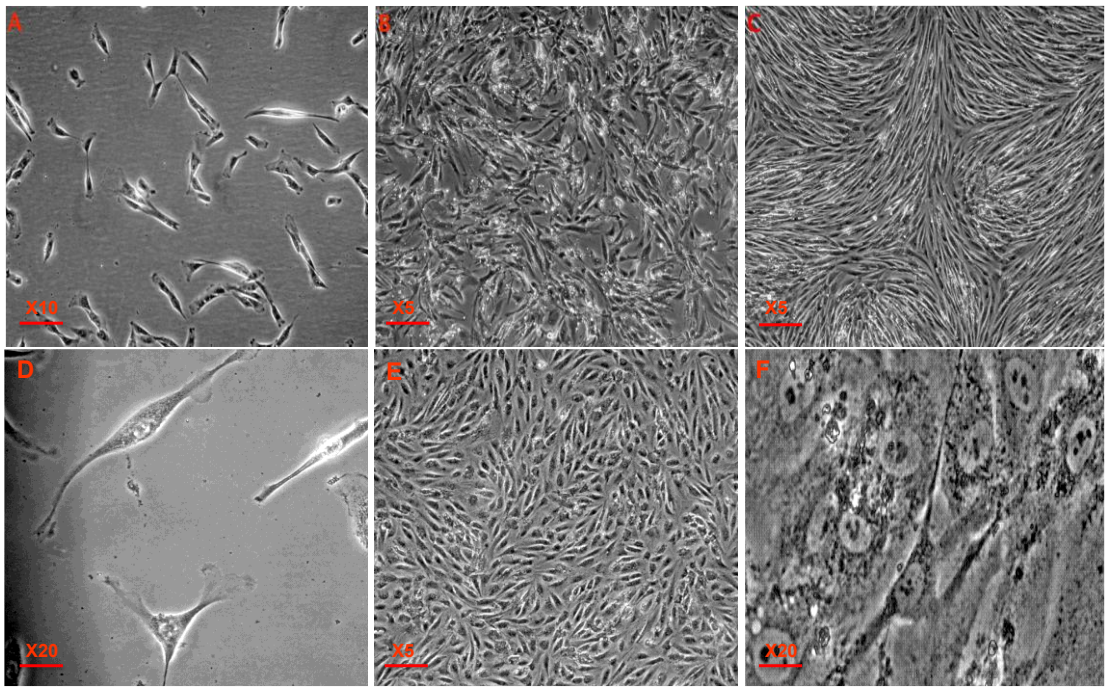


Figure 33 - Cultured human skeletal muscle; A&D) Isolated human myoblasts from the skeletal muscle biopsy (1-week-old); B&E) Human myotubes (under differentiation); C&F) Human myotubes (fully differentiated)

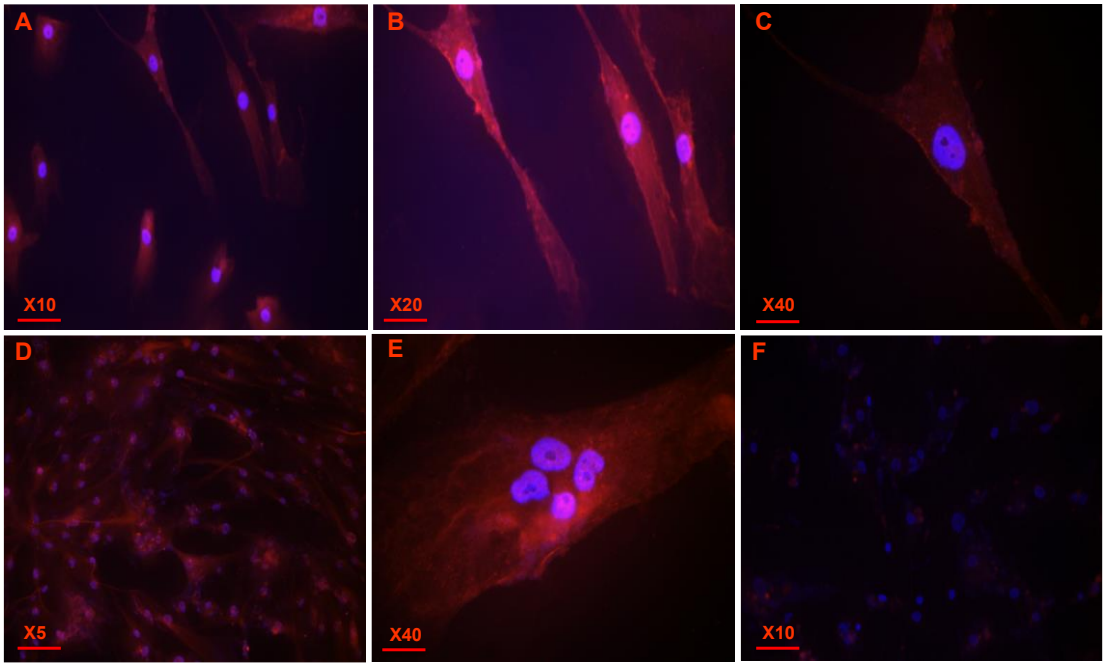


Figure 34 - Cultured human skeletal myotubes showing fluorescent immunohistochemical staining for desmin (Texas red); A, B & C) Isolated human myoblasts from the skeletal muscle biopsy (1-week-old); D) Human myotubes (under differentiation); E) Human myotubes (fully differentiated); F) IgG (negative) control

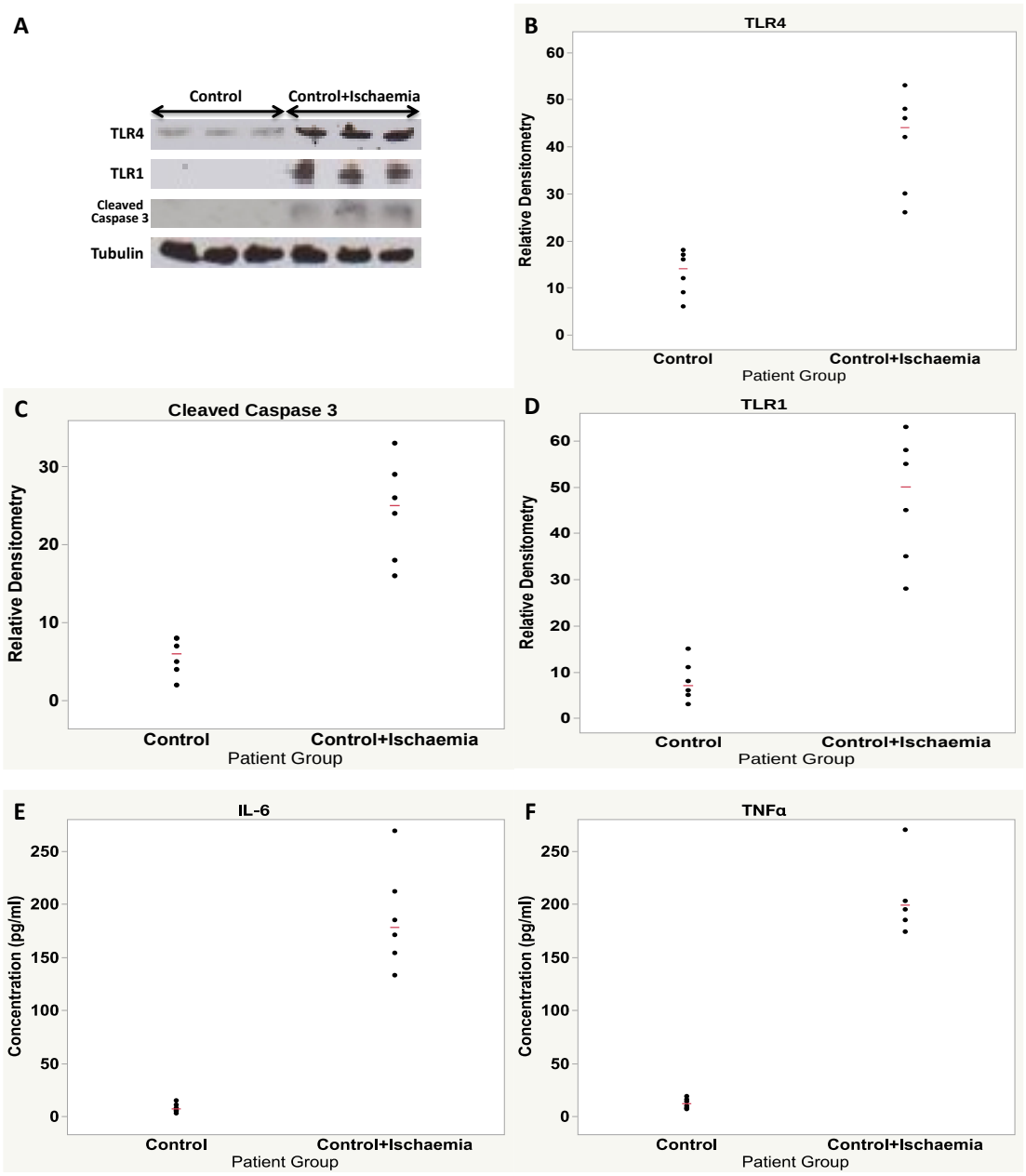


Figure 35 – Effect of ischaemia on myotubes from patients with no PAD A) Representative western blots of TLR4, TLR1 and cleaved caspase 3 B-D) Densitometric analyses of western blots showing upregulation of TLR4, TLR1 and cleaved caspase 3 myotubes cultured in ischaemic conditions (n=6, $p<0.05$) E&F) ELISA data showing increased IL6 and TNFa release by myotubes cultured in ischaemic conditions (n=6, $p<0.05$)

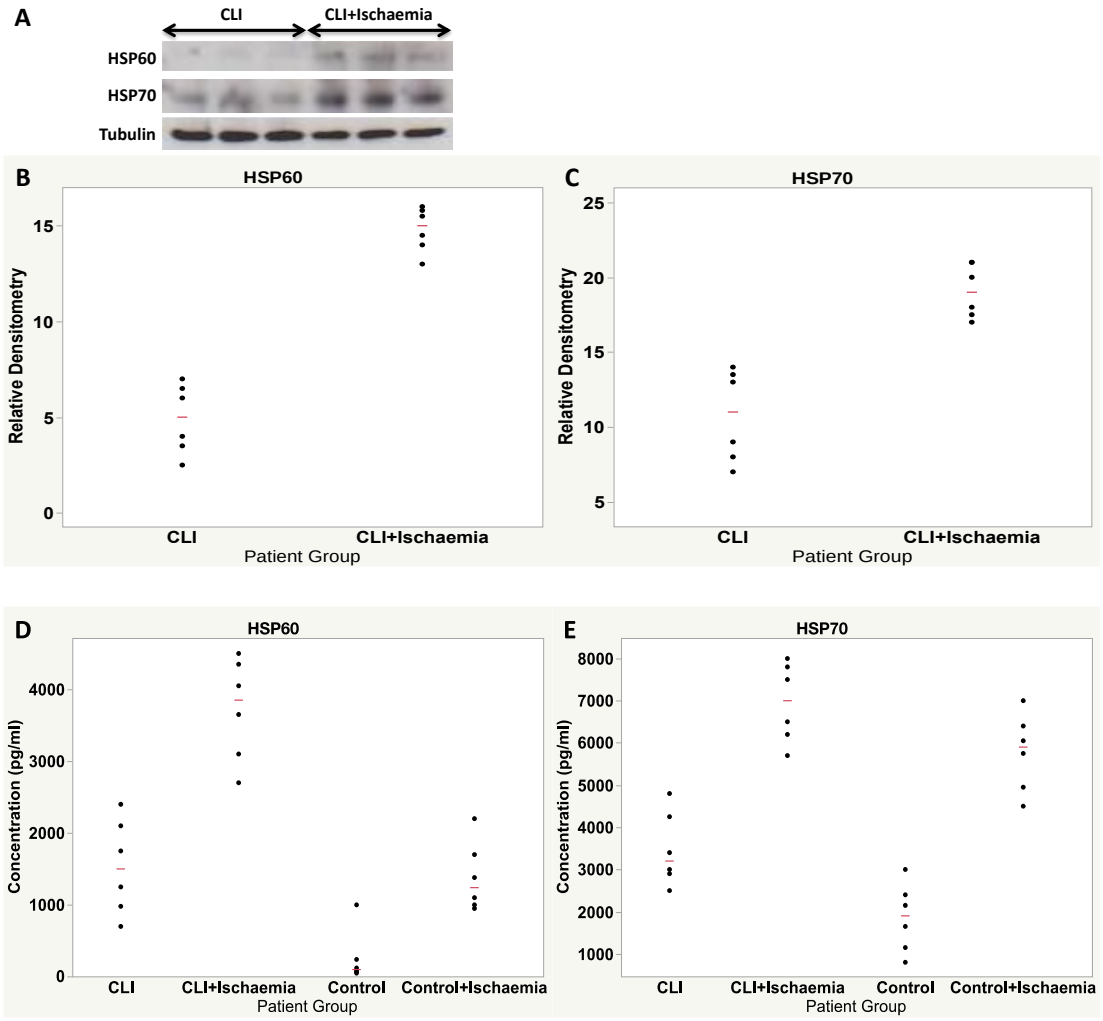


Figure 36 – Effect of ischaemia on myotubes from patients with CLI A) Representative western blots of HSP60 and HSP70 B&C) Densitometric analyses of western blots showing upregulation of HSP60 and HSP70 (n=6, p<0.05) D&F) ELISA data showing increased HSP60 and HSP70 release by myotubes from patients with CLI cultured in ischaemic conditions (n=6, p<0.05)

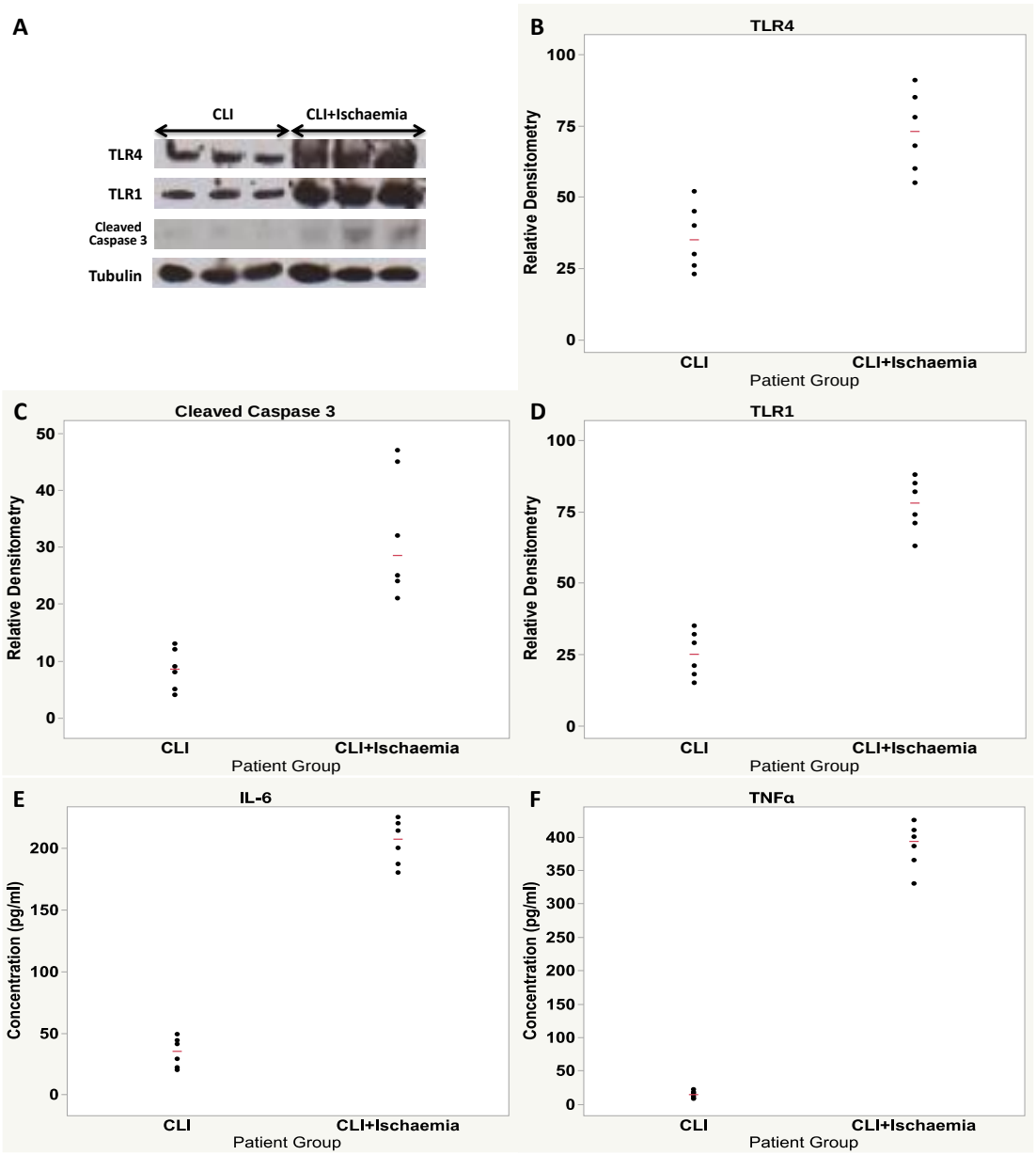


Figure 37 – Effect of ischaemia on myotubes from patients with CLI A) Representative western blots of TLR4, TLR1 and cleaved caspase 3 B-D) Densitometric analyses of western blots showing upregulation of TLR4, TLR1 and cleaved caspase 3 myotubes cultured in ischaemic conditions (n=6, $p<0.05$) E&F) ELISA data showing increased IL6 and TNFa release by myotubes cultured in ischaemic conditions (n=6, $p<0.05$)

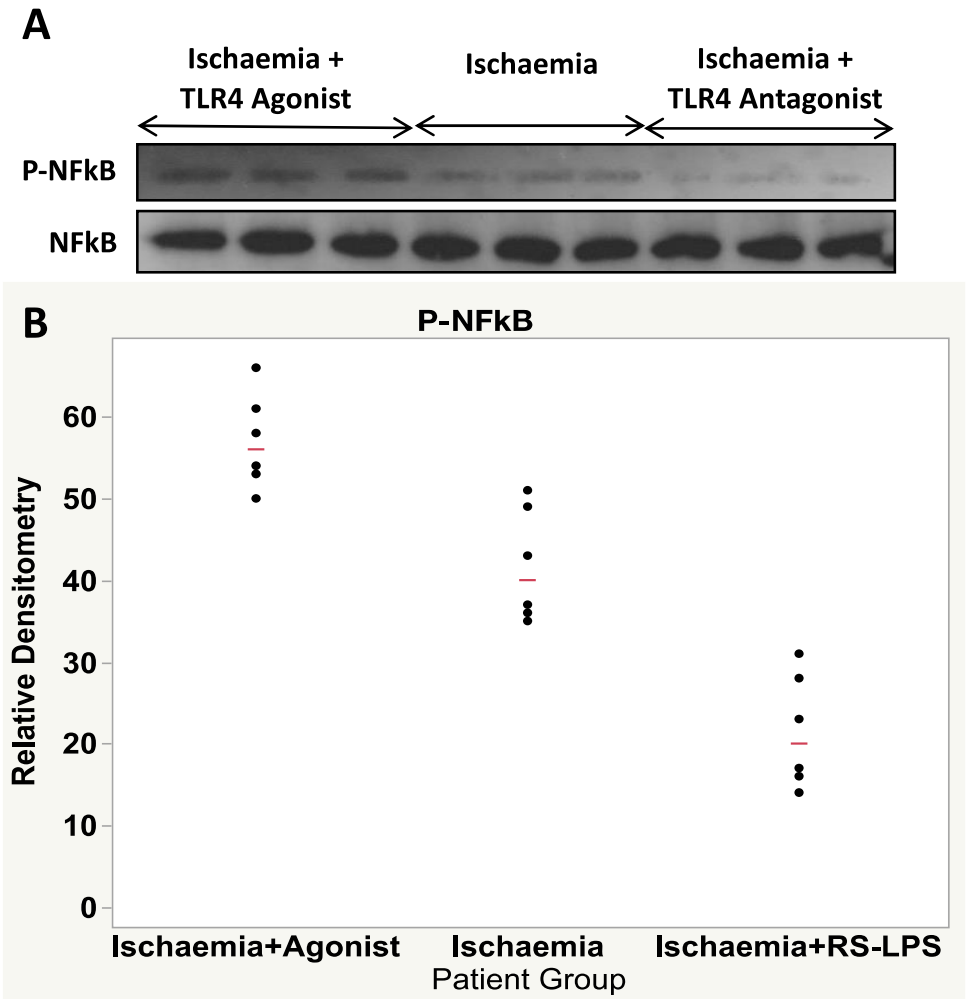


Figure 38 – A) Representative western blots of P-NFkB with TLR4 agonist (synthetic LPS) or TLR4 antagonist (RS-LPS) pre-treatment prior to simulated ischaemia in myotubes from patients with CLI B) Densitometric analyses of western blots of P-NFkB with TLR4 agonist (synthetic LPS) or TLR4 antagonist (RS-LPS) pre-treatment prior to simulated ischaemia in myotubes from patients with CLI (n=6, $p<0.05$)

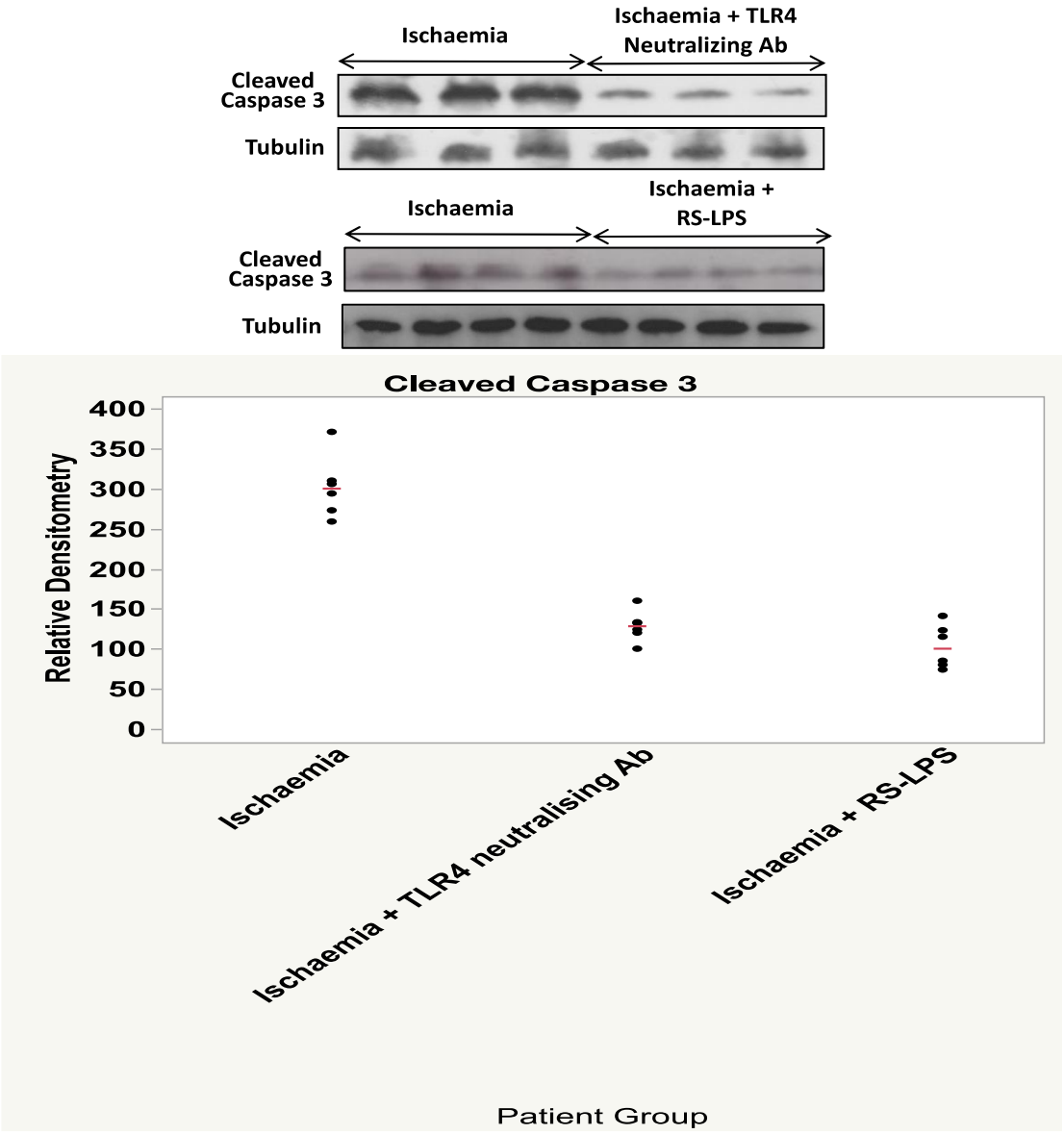


Figure 39 - Western blot analysis (representative blot and densitometric analysis data) of cleaved caspase 3 from patients with CLI which were pre-treated with TLR4 antagonist (RS-LPS) or TLR4 Neutralising Ab prior to simulated ischaemia (n=6, p<0.05)

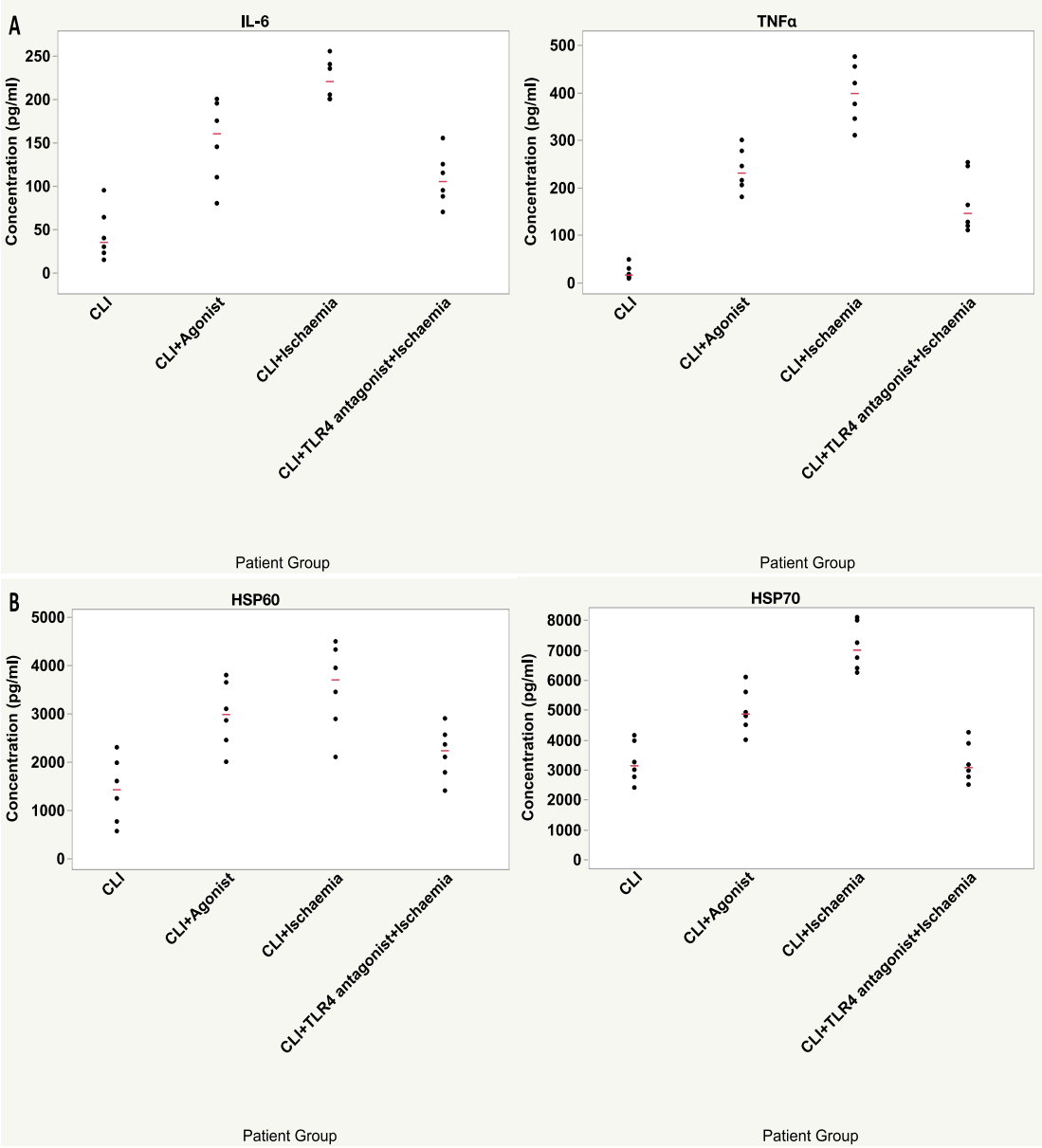


Figure 40 – Effect of TLR4 antagonism and ischaemia on myotubes from patients with CLI A) TLR4 antagonist pre-treatment attenuates ischaemia-induced IL6 and TNFa release (n=6, p<0.05) B) TLR4 antagonist pre-treatment attenuates ischaemia-induced HSP60 and HSP70 release (n=6, p<0.05)

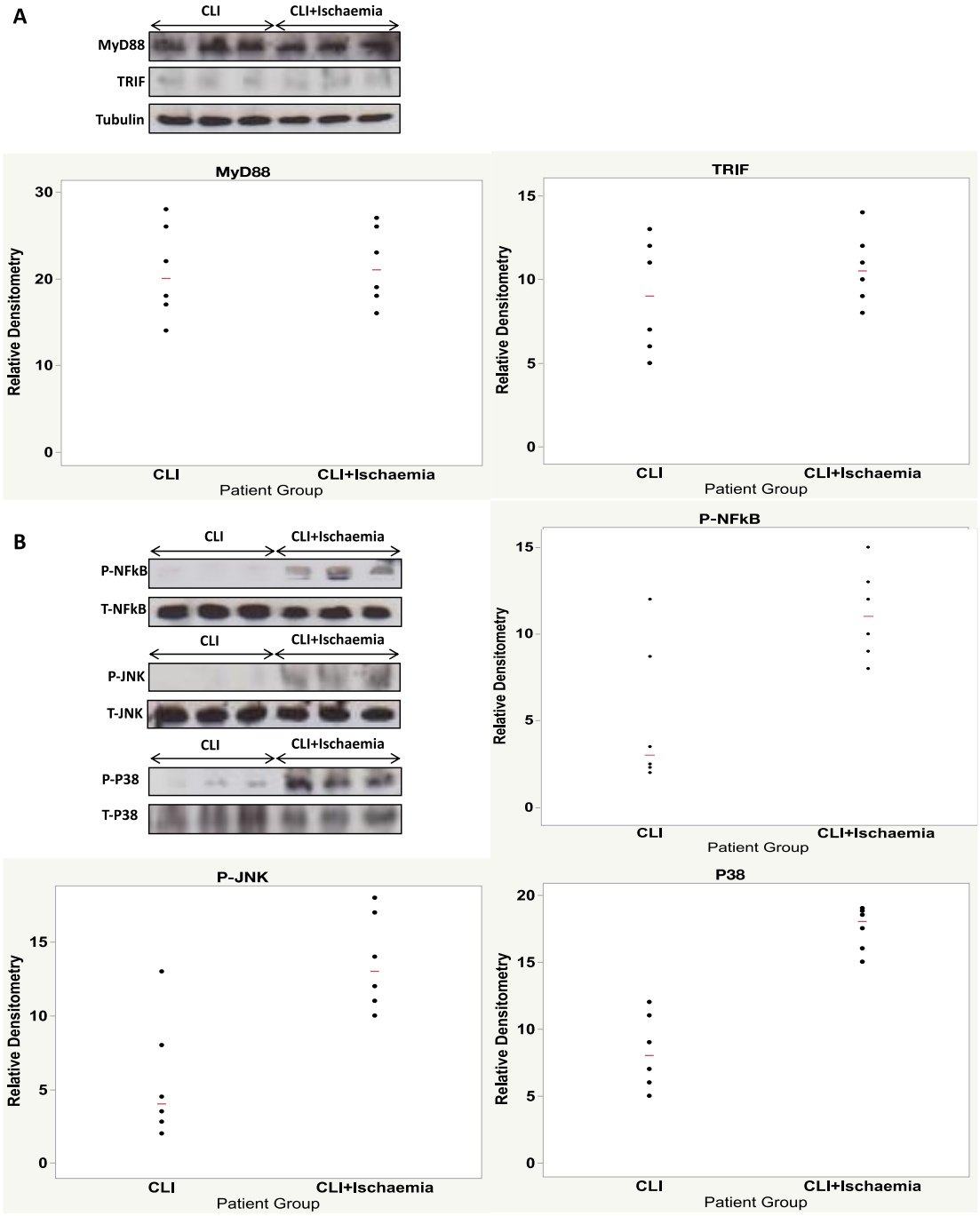


Figure 41 – Effect of ischaemia on myotubes from patients with CLI A) Representative western blots of MyD88, TRIF, and densitometric analyses of western blots showing no significant difference after simulated ischaemia B) Representative western blots of P-NFkB, P-JNK, P-P38, and densitometric analyses of western blots showing upregulation of P-NFkB, P-JNK and P-P38 in myotubes cultured in ischaemic conditions (n=6, $p<0.05$).

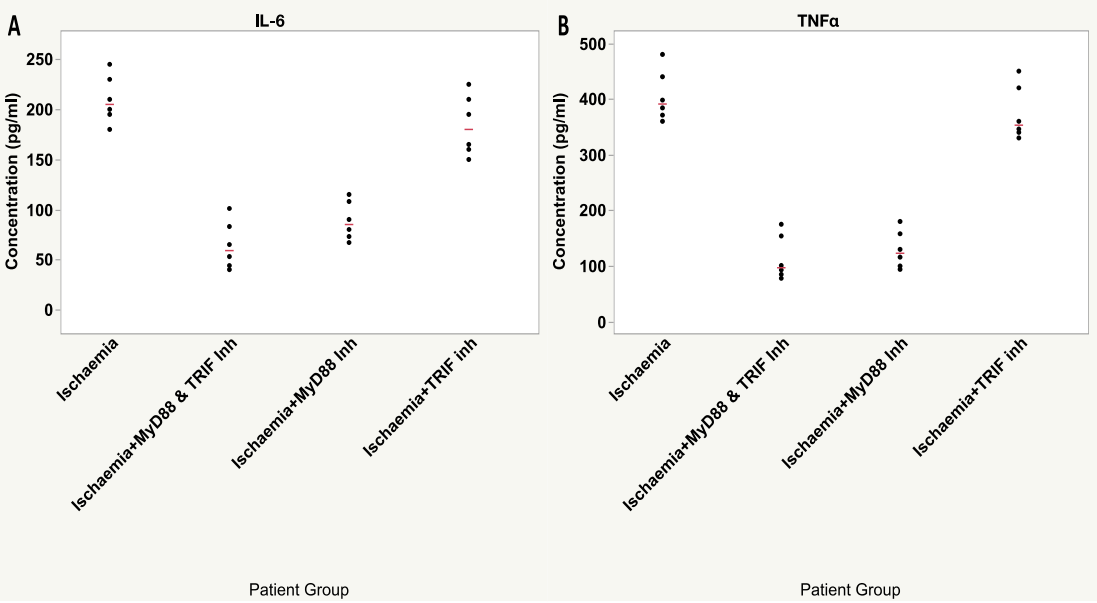


Figure 42 - Representing ELISA analyses of A) IL-6 and B) TNFα in cultured myotubes from patients with CLI, with or without pre-treatment with MyD88 and TRIF inhibitor (n=6, p<0.05)

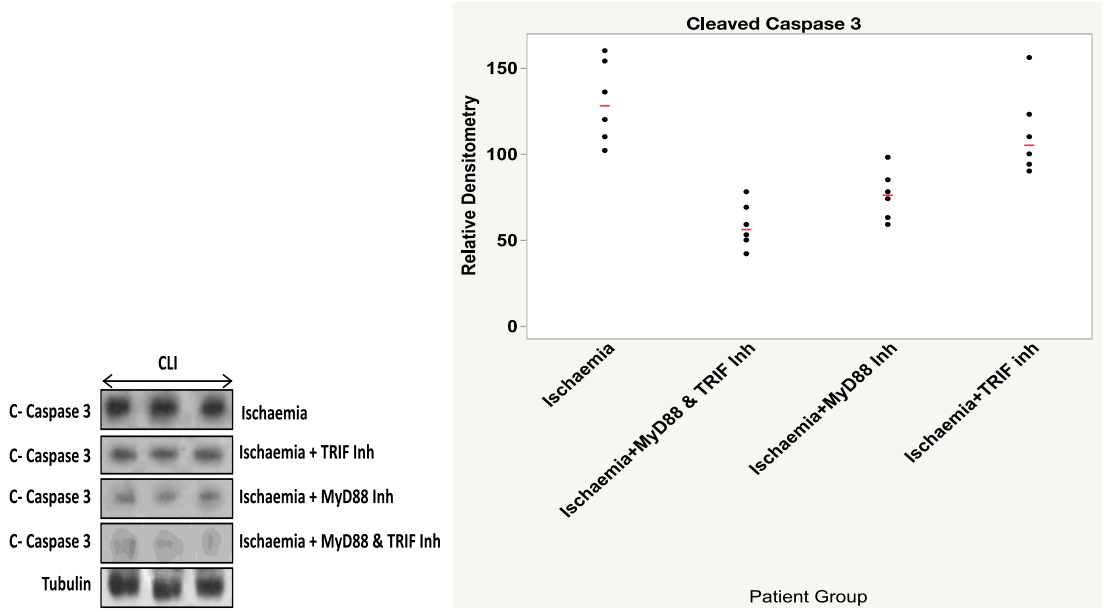


Figure 43 - Effect of ischaemia on myotubes from patients with CLI. Representative western blots of Cleaved Caspase 3 after inhibition of adaptor proteins and densitometric analyses of western blots showing downregulation of Cleaved Caspase 3 after inhibition of MyD88 (N=6, $p<0.05$)

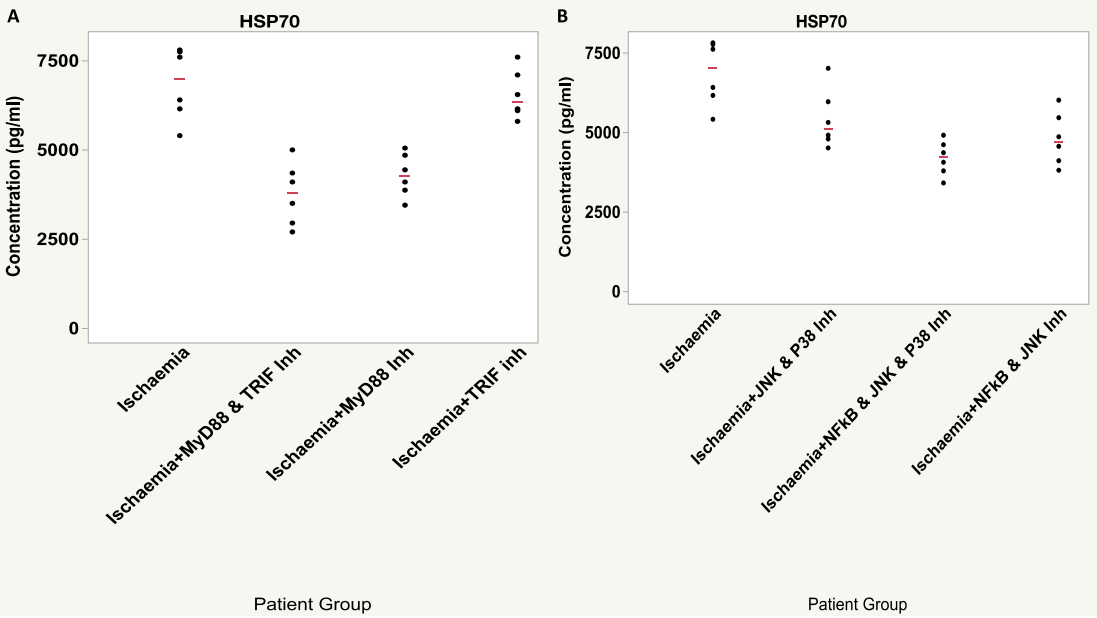


Figure 44 - Representing ELISA analyses of HSP70 in cultured myotubes from patients with CLI, with or without pre-treatment with A) TRIF and MyD88, and B) JNK, NFkB and P38 inhibitors (n=6, p<0.05)

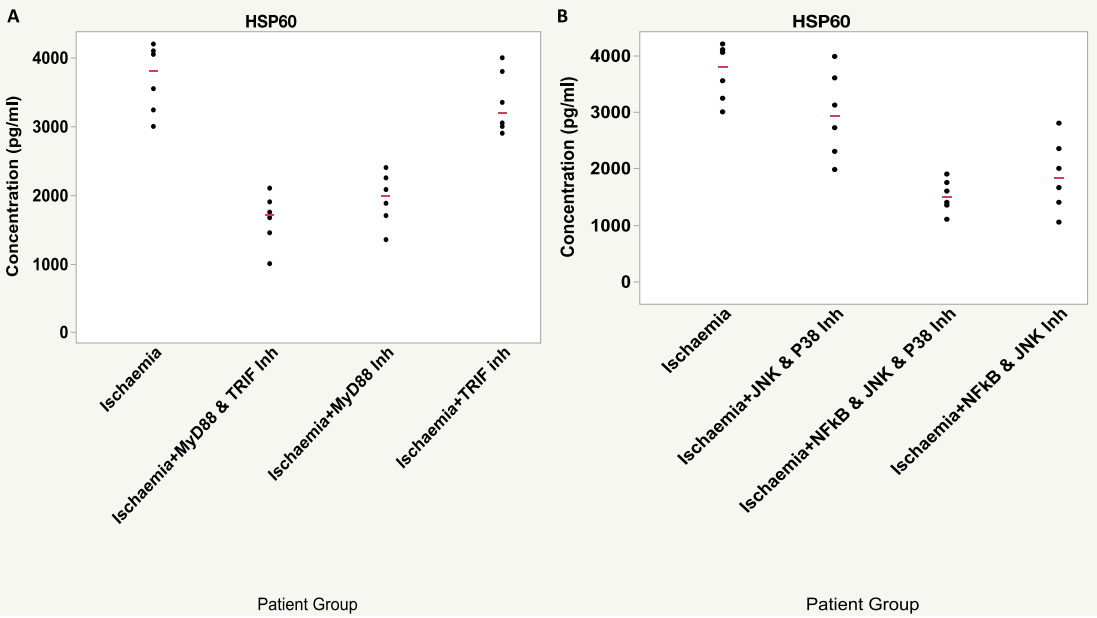


Figure 45 - Representing ELISA analyses of HSP60 in cultured myotubes from patients with critical limb ischaemia, with or without pre-treatment with A) TRIF and MyD88, and B) JNK, NFkB and P38 inhibitors (n=6, p<0.05)

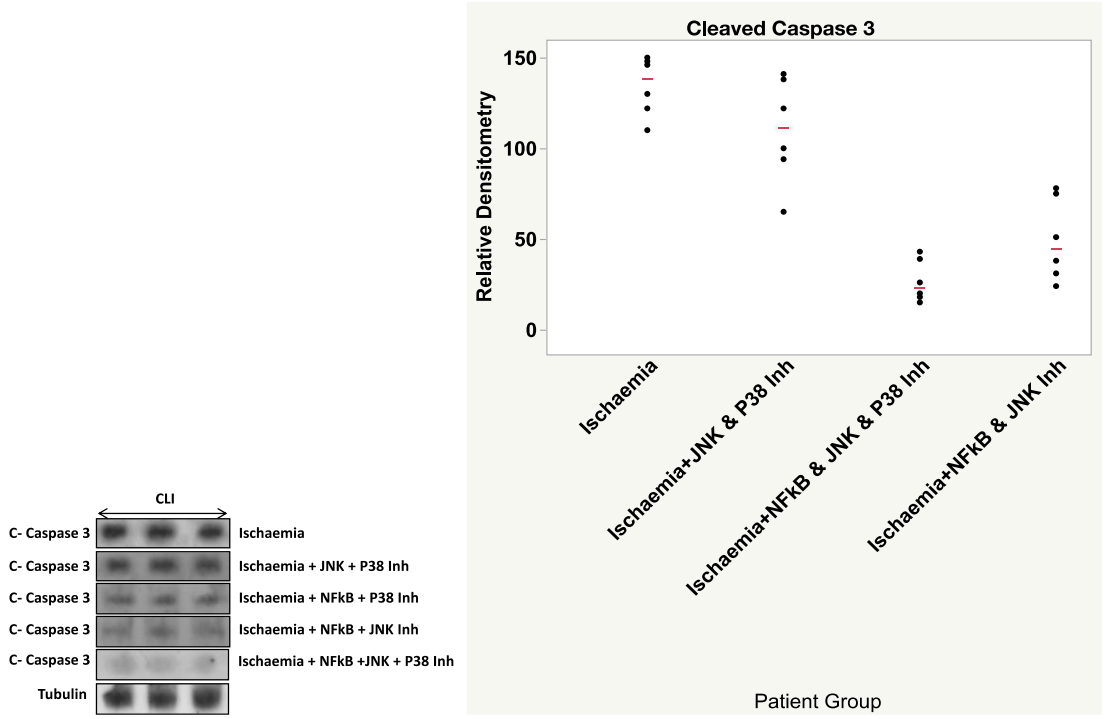


Figure 46 - Effect of ischaemia on myotubes from patients with CLI. Representative western blots of Cleaved Caspase 3 after inhibition of transcription proteins and densitometric analyses of western blots showing downregulation of Cleaved Caspase 3 after inhibition of NFkB (n=6, p<0.05)

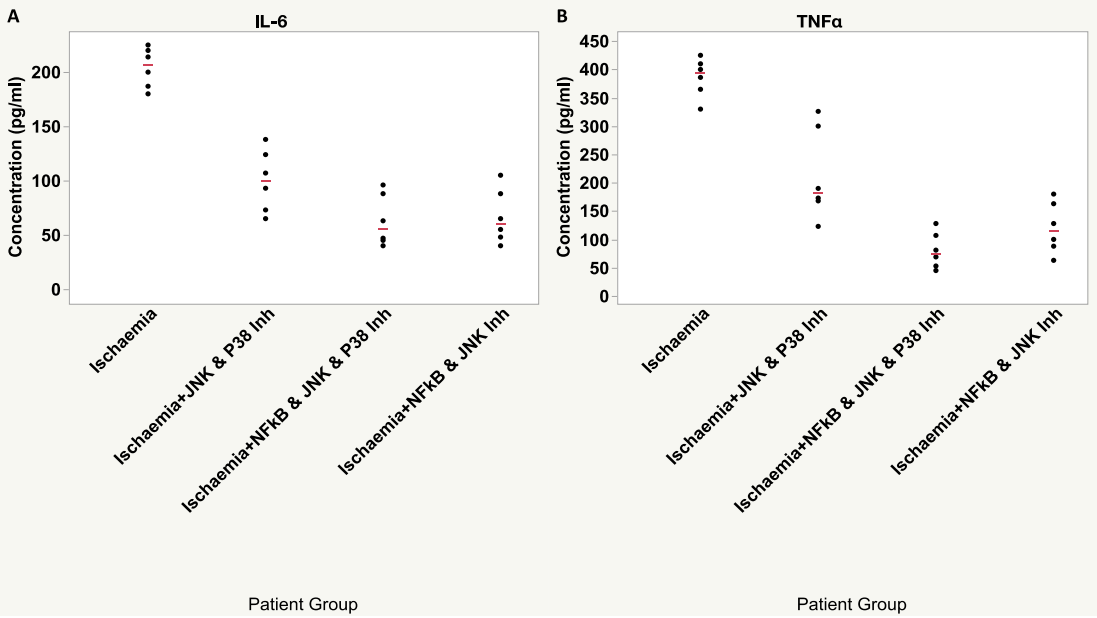


Figure 47 - Representing ELISA analyses of A) IL-6 and B) TNFα in cultured myotubes from patients with CLI, with or without pre-treatment with JNK, NFkB and P38 inhibitors (n=6, p<0.05)

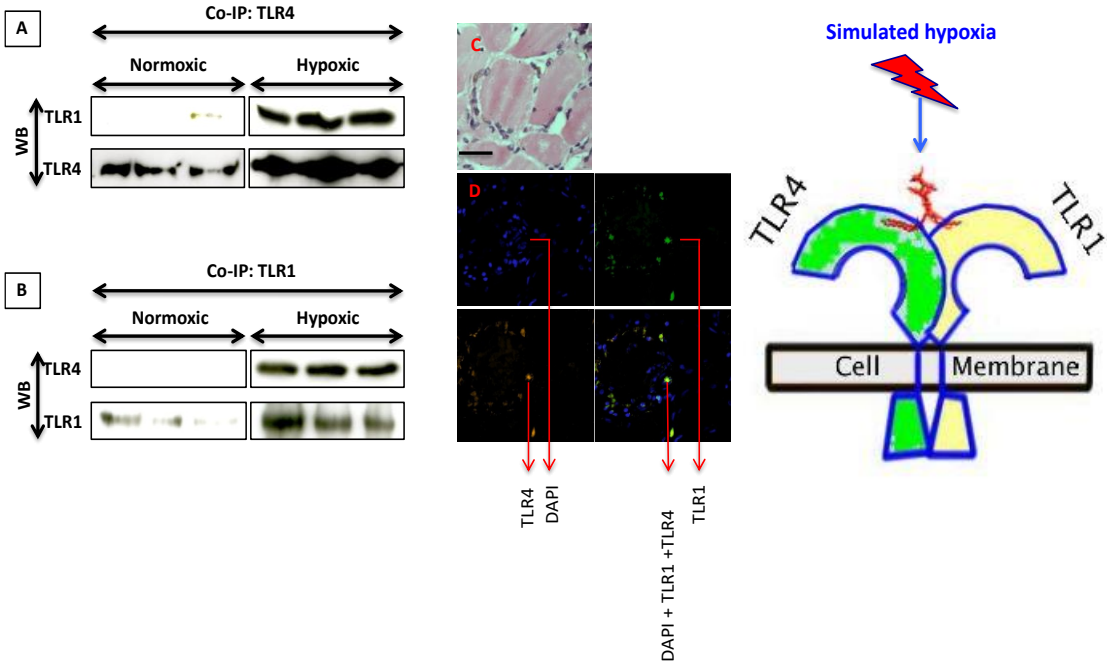


Figure 48 - A) Co-IP with anti-TLR4 and western blot with anti-TLR1 and anti-TLR4 suggesting heterodimerisation of TLR4 & TLR1 B) Co-IP with anti-TLR1 and western blot with anti-TLR4 and anti-TLR1 suggesting heterodimerisation of TLR4 & TLR1 C) H&E staining of ischaemic muscle section (scale bar = 10µm D) Double labelled Fluor-IHC-P staining of TLR1 & TLR4 showing co-localisation of TLR1 & TLR4

CHAPTER 5: *IN VIVO* MODEL OF ISCHAEMIA-INDUCED SKELETAL MUSCLE DAMAGE

5. 1 Introduction

- **5.1.1** Techniques of femoral artery disruption
- **5.1.2** Effect of species
- **5.1.3** Effect of age
- **5.1.4** Acute ischaemia vs. chronic ischaemia
- **5.1.5** Limitations of the current models

5. 2 Aims

5. 3 Experimental design

5. 4 Results

5. 5 Discussion

5.1 Introduction

The utilization of animals as models for human anatomy and physiology was initiated first in ancient Greece (184).

“Ought we, for instance (to give an illustration of what I mean), to begin by discussing each separate species-man, lion, ox, and the like-taking each kind in hand independently of the rest, or ought we rather to deal first with the attributes which they have in common in virtue of some common element of their nature, and proceed from this as a basis for the consideration of them separately?”

-Aristotle (384 -322 BC) “On the Parts of Animals”

Although the early animal modelling was completely observational, it soon became a strong research tool for studying diseases and their treatments.

Animal modelling has its own limitations as animals host different diseases and different responses, however it allows study of *in vivo* responses, often in controlled conditions before translation of experimental findings into clinical trials in humans.

In clinical physiology and phenotypic mimicry models, the pathogenesis of a disease is triggered in an animal model to produce similar phenotypic outcome that is seen in clinical scenarios in human. For example, mechanical disruption of the vasculature and blood flow in an animal model mimics perfusion-related abnormality and dysfunction (185). Further motivation to advance animal modelling resulted in the introduction of transgenic and gene knockout models to investigate the pathologies which were not available in natural models. In transgenic models, an exogenous gene is injected directly into cells at early embryonic stage in animal development. Whilst, in gene knockout models, an endogenous gene is deleted or modified leading to the generation of animals deficient in specific gene of interest. Genetic manipulation was initially introduced in a mouse model in 1980s when the ability to engineer the mouse genome had become increasingly refined (186).

Since then, genetic manipulation has been established in different species such as rats, cats, dogs, pigs, sheep, goats and chickens, however the mouse continues to be the most commonly used for biomedical research. Phenotypic mimicry animal models in genetically manipulated animals have increased the ability to investigate the therapeutic potentials of novel treatments in pre-clinical studies.

Mice are widely used because they are small, prolific, cost effective, easy to maintain and we have the potential to manipulate their genetics. Further, the genetic and physiological similarities between mice and human have attracted considerable attention on mice as potential models for human health and disease (187).

Many aspects of skeletal muscle biology are remarkably similar between mammals (188). In mouse, as in human, skeletal muscle development is the result of the activation of different myogenic cells, derived from bone marrow. The myogenic cells, myoblasts, undergo myotome formation that is followed by fusion of myoblasts to form the primary fibres. Some of the myoblasts continue to proliferate and differentiate to form secondary fibres. At the same time, a basal lamina begins to form around the fibres and after this stage, the myoblasts are known as satellite cells due to their morphological changes. At the end of post-natal development, satellite cells enter a phase of quiescence but can be activated if the muscle tissue is damaged (189). Satellite cells remain associated with muscle fibres after birth and are responsible for muscle growth and repair throughout life (190). Muscle regeneration is also supported by non-myogenic cells, derived from stem cells. Mesenchymal stromal cells and vascular progenitors have been shown to participate in muscle regeneration by giving rise to myogenic stem cells or by stimulating the activation of the existing satellite cells (191, 192).

In both human and mouse, there are significant differences between the skeletal muscle cells in the head, body and the limbs. This is due to the source of myoblasts and the expression pattern of regulatory genes (193,

194). The homeostasis and function of myofibres depend on their size and contractile properties, and also vascular supply, extracellular matrix composition, and efficacy of neural stimulation. The mechanism to generate energy for the contraction of the myofibres depends on the myofibres type: fast myofibres mainly rely on glycogen metabolism, while slow myofibres depends on mitochondria and lipid oxidation. Thus, the number of healthy mitochondria is essential to maintain muscle function.

Healthy blood supply and capillary network is an imperative factor in skeletal muscle homeostasis to deliver oxygen and nutrients and also to remove waste products. Interruption in blood supply will affect skeletal muscle cell functionality and severe ischaemia will result in cell death and necrosis (195).

The ratio between the slow and the fast fibres in skeletal muscle has a significant role in determining the nature of ischaemia-induced skeletal muscle damage. Slow fibre containing muscle, such as soleus, is more sensitive to ischaemia compared to muscle with predominantly fast fibres, such as gastrocnemius. Gastrocnemius has both oxidative and glycogen metabolism which makes it more suitable for the study of ischaemia-induced damage in skeletal muscle damage in the chronic setting (196).

Another essential element in skeletal muscle homeostasis is the extracellular matrix that plays a key role in the transmission of the contractile characteristics of myofibres to move parts of the body. The generated force within myofibres is transferred across the sarcolemma to the basement membrane and then to the interstitial connective tissue where collagen is responsible for contraction (197).

Following skeletal muscle damage in mouse, as in human, inflammation and myogenesis are required for resolution and healing. The process of regeneration starts soon after the infiltration of inflammatory cells and production of cytokines and enzymes. The inflammatory mediators activate the satellite cells which play a key role in the process of regeneration (192). The activated myoblasts pile up at the site of injury and start proliferation

within 24 hours with peak activity on day 3-4. Their activity reduces significantly by day 7-10. By the end of the third week, the damaged innervation is restored with myofibres increased in size (198).

Ischaemia-induced skeletal muscle damage in mouse, shows similar morphological changes to ischaemic human skeletal muscle. The histomorphological changes include increased disruption of inter-myofibres network, oedema and migration of polymorphonuclear neutrophils and phagocytic invasion (199).

Many murine models of hind-limb ischaemia have been described in the literature. Most of these were developed for the assessment of ischaemia-induced angiogenesis, and to understand the pathophysiology of blood flow recovery after ischaemic insult to the hind-limb. Studies generally involved disruption of blood flow to the hind-limb using a range of techniques to ligate +/- excise segments of the hind-limb arterial tree. Tissue perfusion assessed by laser Doppler, and histological assessment of muscle sections and assessment of necrosis were main techniques of post-surgical assessment. Mice developed reduced perfusion in ischaemic limbs but in general, limb perfusion returned to normal by 4-6 weeks at the latest due to effective collateralisation in mice. Here, a range of models described in the literature will be summarised.

5.1.1 Techniques of femoral artery disruption

A. Simple ligation / electrocoagulation

Limbourg *et al*. described a protocol in 10-12 weeks old BALB/c mice for the assessment of ischaemia-induced angiogenesis. They used adult mice as they have fully developed arterial networks and exclusively used male mice to exclude the confounding effects of cyclic oestrogen on the cardiovascular system seen in female mice (200). In this model, the femoral artery was

ligated immediately distal to the origin of the deep femoral branch to redirect blood flow to the collateral arteries. Although this model is suitable for studying angiogenesis, the extent of ischaemia induced by this procedure was insufficient to cause severe functional or histological changes.

Mulkern *et al*. used C57BL/6 mice with ligation of the unilateral hind-limb vessel under general anaesthesia to induce hind-limb ischaemia. The primary endpoint was to assess if the resultant gait can be validated as a surrogate marker for angiogenesis. The animals underwent gait assessment at day 3, 5, 7, 10, 14, 21 and 28 post surgery. Gait analysis was performed using a Plexiglass tube. Angiography was performed at each time point to image the arterial tree and the corresponding collateral formation. To calculate the amount of collateralization, pixel count measurement was done after the angiography. The results showed significant correlation between collateralization and gait improvement, with mice showing complete recovery from the ischaemic insult within 21 to 28 days (201). Although this model of ischaemia showed reduced limb perfusion, the focus was on ischaemia-induced collateralization and the associated ischaemia-induced muscle damage was not studied. In this publication, no data was provided regarding the age and gender of the mice, and no details regarding the level of arterial ligation and whether the vein was ligated or not were available.

Desposito *et al*. used 10 weeks old male C57BL/6 mice with ligation of right femoral artery under general anaesthesia. The ischaemic changes were assessed at day 3, 7 and 14 post surgery, by quantification of neovascularization. Capillary density was measured by fluorescent staining of endothelial cells in muscle biopsies from gastrocnemius muscle, and was expressed as a percentage of pixels per image occupied by blood vessels. Tissue perfusion was assessed by laser Doppler. The results showed reduction of tissue perfusion, followed by significant improvement in the perfusion and neovascularization by day 14 post surgery (202). Although, the

technique caused significant interruption to the hind-limb perfusion, the post-operative ischaemic changes were only observed for a short period of time.

Borne *et al*. used 10-12 weeks old male C57BL/6 mice with unilateral double electrocoagulation of both femoral artery and iliac artery. The hind-limb perfusion was assessed at day 3, 5, 7, 10, 14, 21 and 28 post surgery. A systemic inflammatory response was demonstrated by increased circulating TNFα levels 24-hour post surgery. Perfusion of the hind-limb almost returned to baseline by day 7 (203). Although the level of ligation was high, up to the iliac artery, the mice only showed reduced perfusion for 7 days before recovering to base line tissue perfusion.

Ouma *et al*. used male BALB/c mice weighing between 20 to 25 grams. The left femoral artery was exposed, isolated from the femoral nerve and vein, and ligated distal to the origin of the deep femoral artery, using a 6.0 silk sutures. Tissue perfusion was monitored with laser Doppler, and muscle necrosis was assessed by H&E staining of adductor muscle biopsies (204). Although the interruption to the hind-limb artery was only by ligation of the femoral artery with no excision, the perfusion ratio was still well below the base line on day 28. The histomorphological changes were only assessed on day 21 when necrosis and inflammatory cell infiltration were observed. The authors did not provide details regarding the age of the mice.

Zhang *et al*. used 8 weeks old female C57BL/6 mice with ligation of the femoral artery proximal to origin of profunda. Tissue perfusion was monitored by laser Doppler, and skeletal muscle damage was examined histologically by identifying apoptotic cells (205). The results showed significant reduction in tissue perfusion post surgery, however muscle damage was only assessed histologically on day 21 post surgery, showing increased apoptosis within the ischaemic skeletal muscle.

Kim *et al*. used 8 weeks old male BALB/c mice. The proximal portion of the femoral artery including the superficial and deep branches were ligated twice with 7-0 silk suture. Laser Doppler measurement showed significant reduction

in perfusion post-surgery with some degree of recovery by day 14 (206). Although reduction in tissue perfusion was shown in this study, the follow up period was only until day 14 post surgery, and there was no assessment of the histomorphological changes included.

Aitsebaomo *et al*. used 10 weeks old Sv129 mice, widely used in the production of targeted mutations due to the availability of multiple embryonic stem cell lines derived from them. The femoral artery was exposed and two 7-0 sutures placed proximal to the origin of the lateral caudal femoral artery. Perfusion was reduced post-surgery with foot and thigh perfusion recovering to its base line by day 8. However adductor muscle perfusion continued to increase over its base line by day 14 (207). H&E assessment of biopsies from the adductor muscles showed inflammatory cell infiltration at day 8 and 21.

Jiang *et al*. used 8-10 weeks old female C56BL/6 mice with ligation of the proximal portion of the left femoral artery and vein including the superficial and the deep branches as well as the distal portion of the saphenous artery with 7-0 silk sutures. All arterial branches between the ligations were ligated. Ischaemic damage was assessed by calculating the ischaemic score (0 = no change, 1 = mild discoloration, 2 = moderate to severe discoloration, 3 = necrosis, 4 = amputation), and perfusion was measured by laser Doppler. Perfusion showed full recovery by day 21 post surgery. The ischaemic score was only calculated until day 7 post surgery, and significantly increased at day 1 with continued gradual increase until day 7 (208). The effect of venous congestion on the outcome was not discussed in this study.

Hourde *et al.* established a mouse model of hind-limb ischaemia where the femoral artery was ligated immediately proximal to the bifurcation from the superficial epigastric artery (209). No evidence of toe, foot, leg necrosis, or toe amputation was observed. Electric nerve stimulation of gastrocnemius muscles showed notable changes as the tetanic forces were significantly reduced by day 7, with significant recovery by day 28 post surgery. Histologically, the H&E staining of the gastrocnemius muscle biopsies

showed some centrally nucleated muscle fibres (10% of the muscle cross-sectional area), as evidence for regenerating muscle fibres [Figure 49].

Tekabe *et al*. used 6 weeks old male C57BL/6 mice. The femoral artery was ligated with two sterile 8/0 non-absorbable silk sutures below the inguinal ligament proximally and just above the bifurcation into the superficial and deep femoral arteries distally (210). Biopsies of anterior tibialis muscle were stained for H&E on day 5 post-surgery, and showed significant infiltration of the inflammatory cells [Figure 50].

B. Ligation and excision

Thompson *et al*. ligated and excised the left femoral artery, vein, and nerve proximal from just distal to the inguinal ligament to the level of the popliteal bifurcation in 8-12 weeks old male C57BL/6 mice. Limb perfusion was assessed by laser Doppler. The angiogenesis characteristics were assessed by quantification of CD31, a marker for endothelial cells, in muscle biopsies (211). This model resulted in significant ischaemia in the hind- limb up until day 28 post surgery. However, it was not clear if any animals were lost due to extensive tissue necrosis. Further, the ligation of the nerve along with the vessels was not justified in this technique. The potential changes due to nerve damage, or venous congestion following excision of the vein were not discussed.

Takeda *et al*. used 10-12 weeks old male C57BL/6 mice with the femoral artery ligated distal to the inguinal ligament and proximal to the saphenous-popliteal bifurcation. After all side branches were occluded by electrical cautery, the femoral artery was isolated and the ligated segment excised. Laser Doppler showed significant reduction in tissue perfusion with recovery to base line by day 28 post surgery. H&E staining of the gastrocnemius muscle showed a mixture of necrotic and fibrotic changes associated with infiltration of inflammatory cells. The mice showed some degree of foot

necrosis described as black toes (212). This model showed ischaemia-induced morphological changes in the gastrocnemius muscle similar to those seen in ischaemic human skeletal muscle.

Reis *et al*. used BALB/c male mice weighing between 20 to 25 grams. The femoral, deep femoral, and popliteal arteries were ligated. The femoral arteries were resected at their origin, just below the inguinal ligament, without injuring veins or the femoral nerve. The mice showed extensive tissue necrosis: in some mice involving the whole limb. H&E staining and morphological assessment of the gastrocnemius muscle showed muscle degeneration and infiltration of adipocytes similar to the changes in patients with CLI (213). This model resulted in extensive tissue damage in some mice but no clear details about the age of the animals were given.

Huang *et al*. used adult male C57BL/6 mice weighing between 18 to 22 grams. The left femoral artery was ligated and excised between the inguinal ligament and its bifurcation into the saphenous and popliteal artery using 7-0 propylene sutures (214). This led to significant ischaemia with tissue loss and foot necrosis in some of the animals, however the experiments were only examined until day 14 post surgery with no histological examination to observe the inflammatory changes. The average score of tissue necrosis was presented as 2.2 where 2 represented below ankle amputation and 3 represented above ankle amputation. Again, the age of the mice was not clear in this study.

Nakamura *et al*. used 10 weeks old male BALB/c mice. The entire left deep femoral artery and vein were ligated at two points with surgical 5-0 silk. The vessels were excised between the two points. The mice were sacrificed at day 3, 7, 14, 21 and 1 year and assessed for capillary density and necrosis. This model of ischaemia was associated with some degree of tissue loss and foot necrosis in most animals. H&E staining showed necrotic changes in adductor muscle biopsies up to day 21 post surgery (215). At 1 year follow up, the mice had fully recovered, with H&E staining of the muscle biopsies

showed normal tissue along with some fibrotic and adipose tissue [Figure 51]. The potential effect of venous congestion after excision of the femoral vein was not discussed in this study.

Park *et al*. used adult male C57BL/6 mice, with ligation and excision of the femoral artery. The mice were assessed on day 1, 3 and 21 with laser Doppler showing significant reduction in perfusion and almost full recovery by day 21. The mice showed some toe necrosis. No histomorphological assessment of muscle changes was done (216). The authors did not provide details regarding the age of the mice.

Hellingman *et al*. investigated different surgical techniques to compare the effect of surgical variations on collateral formation (217). Adult male C57BL/6 mice (10-12 weeks old) were used. They compared four different techniques: 1- Ischaemia was induced by electrocoagulation of the left femoral artery, proximal to the superficial epigastric artery which resulted in complete transaction of the artery and retracting of the two ends 2- Electrocoagulation of the common iliac artery 3- Ligation and excision of the common femoral artery from the inguinal ligament to the popliteal artery with electrocoagulation of side branches. 4- Electrocoagulation of the common iliac followed by the femoral artery [Figure 52]. Tissue perfusion was assessed by laser Doppler and angiogenesis was assessed by immuno-staining of the muscle biopsies for CD31. Blood flow restoration was significantly reduced to 54% after 28 days in the group which had double electrocoagulation of both femoral and iliac artery. This was compared to 100% restoration of the blood flow on day 7 post surgery in the groups with single electrocoagulation of femoral artery or iliac artery. After total excision of the femoral artery, mice had 100% recovery of perfusion after 28 days, whilst those who underwent electrocoagulation of the femoral artery had 100% blood flow recovery within 14 days. Single electrocoagulation of the femoral artery or iliac artery, did not result in any distal necrosis, whilst the double ligation model resulted in three out of 10 mice developing necrosis of one or more toenails. This study showed that the

extent of injury to the vascular tree (single ligation of artery, total excision of artery or double ligation) had greater influence on the pattern of blood flow restoration, than the level of arterial occlusion.

Asano *et al*. used 7 weeks old male BALB/c mice. The proximal left femoral artery was ligated at 2 points 3 mm apart, and the artery between the ligatures was excised. Blood flow was measured with laser Doppler. The functional recovery assessment scored 1 and 2 on day 7 and 14 post surgery, respectively (0=lack of any visible mobility, and 3=mobility equal to non-ischaemic hind limbs, with scores 1 and 2 in between). Histological examination showed that gastrocnemius muscles were atrophic, and the number of nuclei of both muscles and pericytes was decreased. The extracellular space had widened, and capillaries were barely observed [Figure 53]. No apparent necrosis was observed in this model (218).

In conclusion, the above studies suggest that the extent in length of the disruption to the hind-limb arterial tree has more impact on the severity of tissue perfusion reduction and ischaemia-induced tissue damage, than the level of the disruption. Further, the recovery period to restore the baseline tissue perfusion is longer in models with more extensive disruption, providing a longer period for investigators to study the ischaemic changes.

5.1.2 Effect of species

Schaper *et al*. used 11-13 week old male C57BL/6 and BALB/c mice to induce hind-limb ischaemia to compare the collateral vasculature between the two strains. The femoral artery was ligated and limb perfusion was assessed by laser Doppler imaging, visible light oximetry, treadmill testing, electron paramagnetic resonance (EPR) oximetry, x-ray angiography, and histology. Assessment of the tissue perfusion showed less initial ischaemia and then complete recovery in C57BL/6 mice, compared to BALB/c mice that showed more severe initial ischaemia followed by poorer recovery at week 3 post

surgery. The histological examination of thigh muscles showed more prominent ischaemia-induced muscle damage in BALB/c mice described as multiple necrotic and regenerating areas with destroyed myofibres, and myofibres with central nuclei (219).

Scholz *et al.* used 11 week old male BLAB/c and C57BL/6 mice to examine the mechanisms of restoration of the tissue perfusion in hind-limb ischaemia. The femoral artery was ligated and the tissue perfusion was assessed by laser Doppler at day 3, 7 and 21 post surgery. The perfusion recovery was quicker in the C57Bl/6 group, where it reached more than 80% of control values at day 7 post surgery. At that time BALB/c mice showed only 20% recovery. Cell damage in the gastrocnemius muscle was assessed as the number of nuclei that had taken up propidium iodide per 100 muscle fibres. It was 15+/- 5 in C57BL/6 mice and 71+/-14 in BALB/c mice ($P<0.01$). In non-operated hind-limbs it was less than 1 for both groups (220).

The above studies highlight the importance of the genetic background and the existing collateral circulation when designing a model of hind-limb ischaemia. C57BL/6 mice showed less severe reduction in tissue perfusion post surgery, but severe enough to cause ischaemia-induced inflammatory changes in the skeletal muscle, compared to BALB/c mice which showed more severe tissue damage with poorer tissue perfusion recovery.

5.1.3 Effect of age

Rey *et al*. used male C57BL/6 mice to induce ischaemia by complete ligation and excision of the left femoral artery and its branches. Limb perfusion was assessed by laser Doppler, and compared in two groups of mice aged 12 weeks and 13 months. The younger mice showed complete blood flow recovery by day 14 post surgery, whereas the older mice did not show complete blood flow recovery until day 28. In addition, older mice had higher

motor impairment scores and limb necrosis rates than the younger mice (221).

Bosch-Marce *et al*. used male C57BL/6 mice. After ligating the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated, and the artery and all the side branches were dissected free; after this, the femoral artery and attached side branches were then excised. Blood flow recovery in the ischaemic limb was compared in 2, 8 and 20 months old mice for a period of 35 days post-surgery. The blood flow recovery was most impaired in 20 months old mice followed by 8 months old mice, when compared to the young 2 months old mice. Further, the older mice showed less HIF-1α levels in the ischaemic limb compared to the younger mice (222).

The results of the above studies suggest that aging is associated with impaired tissue perfusion recovery and increased ischaemia-induced tissue damage. Unlike younger mice, which recover spontaneously with no permanent damage, the older mice may represent a better model of hind-limb ischaemia more similar to patients with CLI. However, aging is a multifactorial component which can be a confounding factor in the overall physiology of the animal.

5.1.4 Acute ischaemia vs. chronic ischaemia

Yang *et al.* described a mouse model of hind-limb ischaemia with gradual arterial occlusion. The authors argued that acute arterial occlusion results in sudden increase of shear stress within the existing collaterals. The upregulation of shear stress responsive and inflammatory genes in the region of collaterals promote the process of angiogenesis, compromising the study of tissue response to ischaemia only. In this study, 3-5 months old C57BL/6 mice were used. Acute ischaemia was induced by excision of femoral artery and ligation of the side branches, whilst the gradual ischaemia was induced by ligating all branches of the left femoral artery and then placing ameroid

constrictors (0.25 mm internal diameter) around the proximal and distal femoral artery. Ameroid constrictors have an outer metal sleeve encasing an inner layer of a hygroscopic material which induce gradual vessel occlusion as they absorb moisture from the surrounding tissues. The tissue perfusion recovery was assessed by laser Doppler at day 1, 7, 14 and 35 post surgery, and the H&E staining was carried out to assess the muscle necrosis and inflammatory cell infiltration at day 3 post surgery. The analysis of tissue perfusion results showed rapid deterioration in the acute model soon after the surgery with faster recovery to almost baseline at day 35, compared to more gradual decrease in tissue perfusion after surgery with slower recovery rate even at day 35 post surgery. The H&E examination of the ischaemic gastrocnemius muscle showed greater necrosis with infiltration of inflammatory cells in the acute model, compared with no significant necrosis or inflammatory cell infiltration in the gradual model (223). In this study, the ischaemia-induced morphological changes in the skeletal muscle was only assessed at day 3 post surgery. Further, the lack of significant inflammatory cell infiltration in the gradual model may suggest that the induced-ischaemia is not severe enough to mimic the skeletal muscle damage observed in patients with CLI.

Padgett *et al*. recently described another model of gradual ischaemia in BALB/c mice, compared to a model of acute ischaemia. In this study, the acute ischaemia was induced by double ligation of the femoral artery, whilst the gradual ischaemia was induced by placing ameroid constrictors at proximal and distal parts of the femoral artery, with side branches left intact. The authors showed less disruption in tissue perfusion in the gradual model post surgery (224). However, the perfusion was only assessed immediately post surgery with no further follow up. Further, there was no histological examination to assess and compare the ischaemia-induced skeletal muscle damage.

The above studies suggest that models of gradual ischaemia represent changes more similar to the changes in CLI. Gradual ischaemia avoids the unnecessary activation of shear stress-induced genes, hence less extensive inflammatory response in the skeletal muscle. However, the application of these gradual models is more challenging technically and less cost effective.

5.1.5 Limitations of the current models

Most of the current hind-limb ischaemia models are designed to represent and assess the changes in the haemodynamics with primary endpoint of blood flow restoration after the induced ischaemia. We know from clinical studies that the functional outcomes in patients with CLI is not only dependent on blood flow restoration (225, 226). An animal model of hind-limb ischaemia which recapitulates the changes in skeletal muscle similar to the changes in CLI, is required for testing strategies to improve muscle dysfunction.

Results from the different studies are not comparable due to differences in technique, species and age. Some studies did not give clear information on age, species, or the number of animals that did not survive or experienced severe ischaemia. Also, in some studies, lack of clear information on the surgical procedure regarding the instruments, sutures, dressing, type of anaesthesia or the post operative management render them less reproducible. Most models only induce a short period of ischaemia, providing a limited period to study the ischaemia-induced changes. Further, early tissue perfusion recovery compromises the investigators ability to study the chronic effects of -ischaemia. This can be the result of recruitment of existing collateral vessels, and the ability to do so shows inter-strain and inter-animal variability (227, 228).

5.2 Aims

The aim of this chapter was to develop a model of hind-limb ischaemia that induced changes of the affected muscle which were more comparable to the changes in chronic limb ischaemia in patients with CLI, to aid further studies in this area and to evaluate potential novel therapies.

Objectives:

The objectives were:

1) To assess histomorphological changes in critically ischaemic human skeletal muscle
2) To develop a murine model of hind-limb ischaemia which demonstrates changes similar to those that occur in skeletal muscle of patients with CLI.

Hypothesis:

It was hypothesized that:

- Excision of common and superficial femoral artery with ligation of the side branches in mice of different strain/ age would alter the resulting muscle changes.

5.3 Experimental design

Changes in critically ischaemic human skeletal muscle were assessed in gastrocnemius biopsies taken from patients undergoing major lower limb amputation due to CLI (n=4). Histomorphological changes were assessed using H&E staining.

In order to identify a murine model which best recapitulates these changes, hind-limb ischaemia was induced by excision of common and superficial

femoral artery and ligation of the side branches (Appendix 1) in BALB/c and C57BL/6 mice of 6 and 12 weeks of age (n=10 in each group) [Figure 54]. We only used male mice to exclude the confounding factor of cyclic oestrogen. To investigate the muscle damage, gastrocnemius and tibialis anterior muscles were biopsied. These muscles are both distal enough to sustain severe ischaemia, and consist of both slow and fast fibres which respond to the ischaemia with both aerobic and anaerobic mechanisms.

All procedures were performed in accordance to Home Office legislation, under Project Licence number 70/7087. ARRIVE guidelines were considered and followed to improve the design, analysis and reporting of the *in vivo* experiments (229).

The mice were kept pathogen-free in groups of 6 in a strict 12-hour night and day cycle. They were fed standard chow with free access to water. Animals were anesthetized by intraperitoneal injection of 2% Avertin, 2, 2, 2-Tribromoethanol, (0.25 ml/g). To maintain the anaesthesia, 1% isoflurane mixed in 100% oxygen with flow of 0.8 l/min was used during the procedure [Figure 55]. All the operations were done over two consequent days during the day time by same operator.

After the mouse was anaesthetized, the hair on the abdominal wall, pelvis and the upper legs was shaved using an electronic shaver. The skin was cleaned using alcohol spray. A skin incision was made on the upper thigh of the mouse. The inguinal ligament and the upper half of the femoral artery were exposed using sharp and blunt dissection. The femoral artery was ligated with sterile 7-0 Prolene suture at the level of inguinal ligament proximally and just above the popliteal artery distally. All the side branches were ligated with 7-0 Prolene suture [Figure 56]. The skin incision was closed with 5-0 silk suture. Animals were nursed for a period of one hour post surgery in a recovery area on a 37^0C warming pad, with buprenorphine (0.1 mg/kg, SC) to manage the pain.

Tissue perfusion and evidence of morphological changes and damage within the ischaemic muscle were assessed at days 1, 3, 7, 14 and 21 by laser Doppler (Moor Instruments) and H&E staining, respectively (Chapter 2). The ischaemic hind-limb perfusion was described as a ratio of the color-coded perfusion image of the ischaemic limb to the non-ischaemic one.

Further, H&E staining of harvested lung, liver, heart and kidney were carried out to investigate the associated systemic inflammatory response. Inflammatory cell quantification was carried out using ImageJ 1.4s (US National Institutes of Health) on H&E stained paraffin embedded left hind-limb skeletal muscle sections.

5.4 Results

Consistent with previous reports, muscle sections from patients with CLI demonstrated features of muscle fibre injury, including loss of muscle fibres with concomitant fibroadipocytic changes, as well as variation in muscle fibre size and evidence of degeneration and necrosis [Figure 57].

Laser Doppler images of the ischaemic hind-limb showed reduced perfusion soon after surgery, with blood flow restoration near to baseline by day 21 in all three groups (BALB/c 12 weeks old, day 1, median 0.15, range 0.10-0.18; day 21, median 1.08, range 0.95-1.15; BALB/c 6 weeks old, day 1, median 0.18, range 0.12-0.20, day 21, median 1.05, range 0.90-1.10; C57BL/6 12 weeks old, day 1, median 0.10, range 0.08-0.18, day 21, median 1.10, range 0.99-1.18) [Figure 58].

Histological assessment of the muscle biopsies demonstrated a similar pattern of histomorphological changes in both C57BL/6 and BALB/c strains at day 7, 14 and 21 including inflammation, degeneration, fibrosis, adiposis and necrosis. However younger animals showed less severe muscle damage, and faster resolution of muscle injury by day 21 post-surgery.

The inflammatory cell infiltration was significantly less in younger mice at day 21 post-surgery (BALB/c 6 weeks old, median 9%, range 4-14%; BALB/c 12 weeks old, median 14%, range 9-19%; C57BL/6 12 weeks old, median 15%, range 12-19%) (P value < 0.05, oneway ANOVA test). [Figure 59, Figure 60, Figure 61, Figure 62].

The H&E staining of the gastrocnemius and tibilais anterior muscle sections were found to be similar in terms of demonstrating the features of ischaemia-induced skeletal muscle damage: degeneration, regeneration, fibrotic tissue, adipose tissue and necrosis [Figure 59, Figure 60, Figure 61, Figure 62]. Further, H&E analyses of the lung biopsies showed inflammatory cell infiltration in the lung tissue suggesting systemic inflammatory response; however there was no obvious inflammatory changes observed in biopsies from heart, kidney and liver [Figure 63, Figure 64, Figure 65].

5.5 Discussion

The previously established mouse models of hind-limb ischaemia were mainly focused on the effect of blood flow interruption and perfusion changes on the arterial tree and angiogenesis.

The role of ischaemia-induced myopathy in the poor functional outcomes of patients with CLI is increasingly recognised, however, the detailed mechanisms involved are not fully understood. Accordingly, for the purpose of this study, we required a mouse model that mimicked the skeletal muscle changes seen in ischaemic human muscle.

The extent of the arterial injury is associated with different patterns of perfusion restoration (230). Excision of common and superficial femoral arteries with ligation of the side branches in our model in male C57BL/6 mice of 12 weeks of age resulted in reproducible ischaemia-induced skeletal muscle changes that closely mimicked those that occur in human skeletal muscle in CLI. The extensive excision of the femoral artery might have

affected the post-surgery angiogenesis, but in terms of the skeletal muscle damage, it was more desirable.

Westvik et al. showed that young adult and aged mice had a similar degree of decreased perfusion soon after interruption of the lower limb arterial tree, however the young mice recovered faster whilst the aged mice had worse functional recovery (231). Genetic differences in pre-existent collateral vasculature can profoundly affect outcomes by compensatory collateral artery growth in different mouse strains (219). McClung et al. showed that C57BL/6 and BALB/c mice displayed strain-specific differences in myofibres responses after hind-limb ischaemia, with significantly greater myofibre atrophy, greater apoptosis, and attenuated myogenic regulatory gene expression and stress-responsive signalling in BALB/c mice (232).

In agreement with our findings, previous reports showed that the severity of the ischaemia increases with the age of the mouse, whilst BALB/c strain manifests more tissue necrosis after the same experimental procedure, compared to C57BL/6 mice (219).

In our observations, the ischaemia-induced histomorphological changes in skeletal muscle in 12 weeks old mice were more similar to what we observed in skeletal muscle from patients with CLI, compared to those in 6 weeks old mice. C57BL/6 mice showed more reliable pattern of tissue perfusion restoration which may be the result of variations in pre-existing collateral vasculature in the BALB/c strain mice (233). Further, we did not observe any obvious histological difference between gastrocnemius and tibialis anterior muscle, in terms of the response to the induced ischaemia. Based on our findings we decided to proceed with our *in vivo* work by excising the common and superficial femoral artery and ligating the side branches in 12 weeks old C57BL/6 mice.

Limitations:

The chronic models of ischaemia with gradual occlusion were not tried in our experiments, partly due to limitations in financial resources and time but also limitations imposed by our project licence.

Since patients with CLI are usually elderly, it might worth considering the model of hind-limb ischaemia in elder mice; however, again it would be expensive and also difficult to maintain.

Further, in our experiments, we did not perform physiological assessments, as we mainly used histological changes as outcome measure.

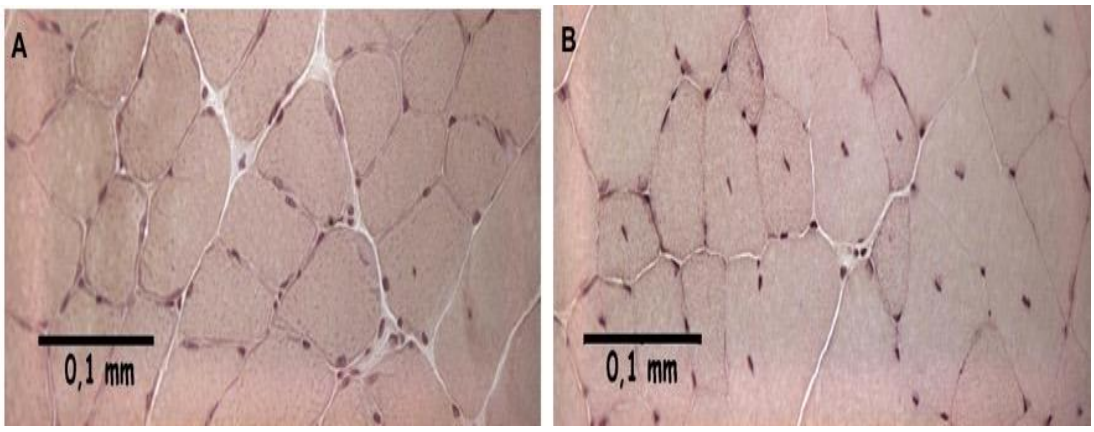


Figure 49 – H&E staining of gastrocnemius muscle biopsies from Hourde *et al.* A) normal muscle B) ischaemic muscle (209)

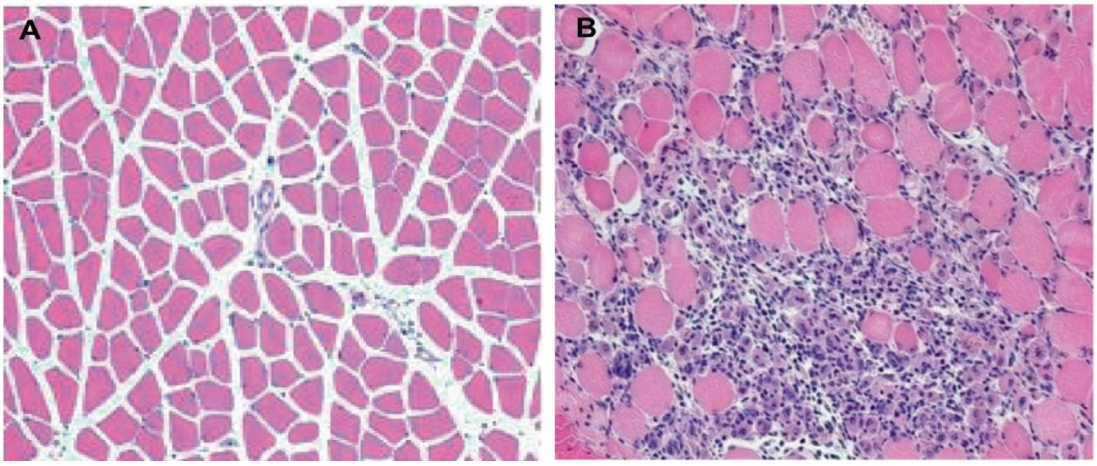


Figure 50 - H&E staining of gastrocnemius muscle biopsies from Tekabe et al. A) normal muscle B) ischaemic muscle (210)

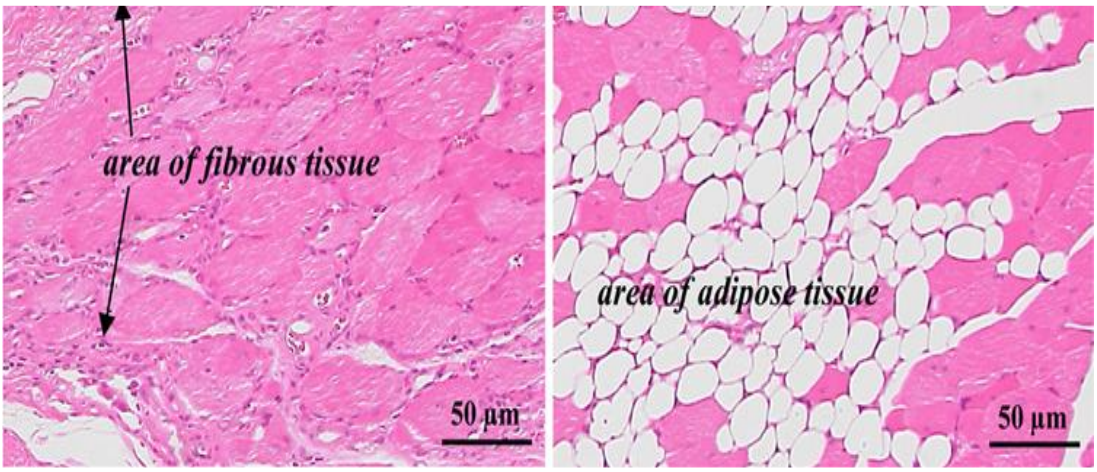


Figure 51 – From Nakamura *et al*. showing fibrotic and adipose tissue in muscle biopsies one year post excision of the femoral artery and vein (215)

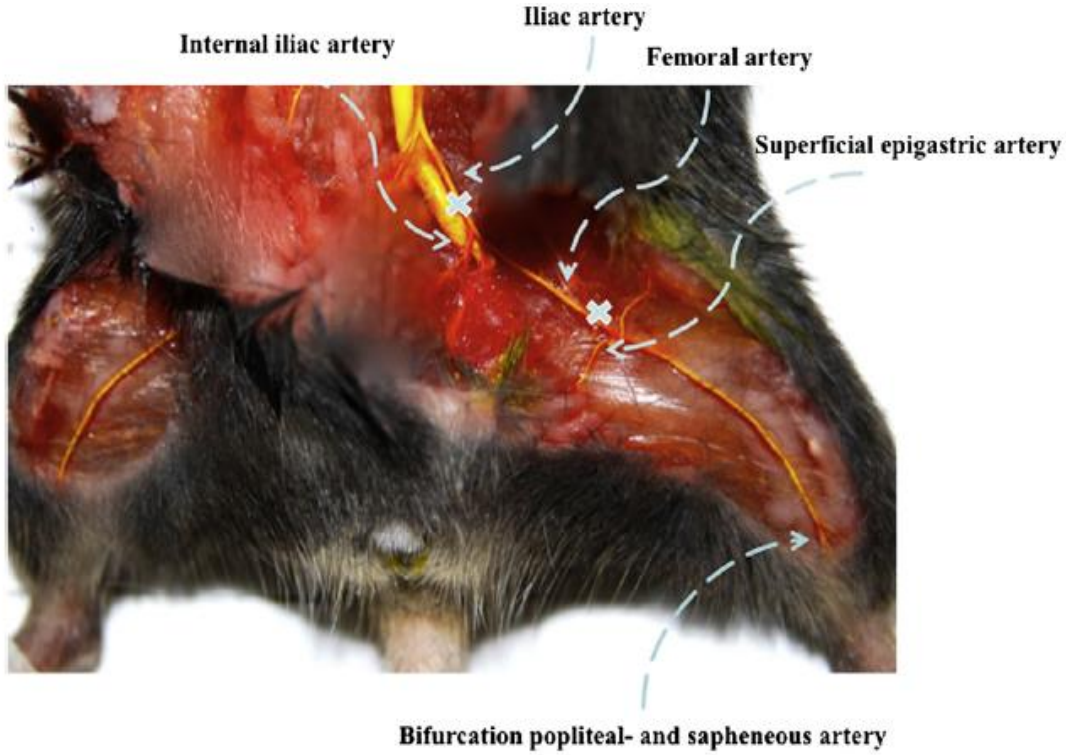


Figure 52 – Hellingman *et al*. compared 4 different surgical approaches of hind-limb ischaemia in mice (217)

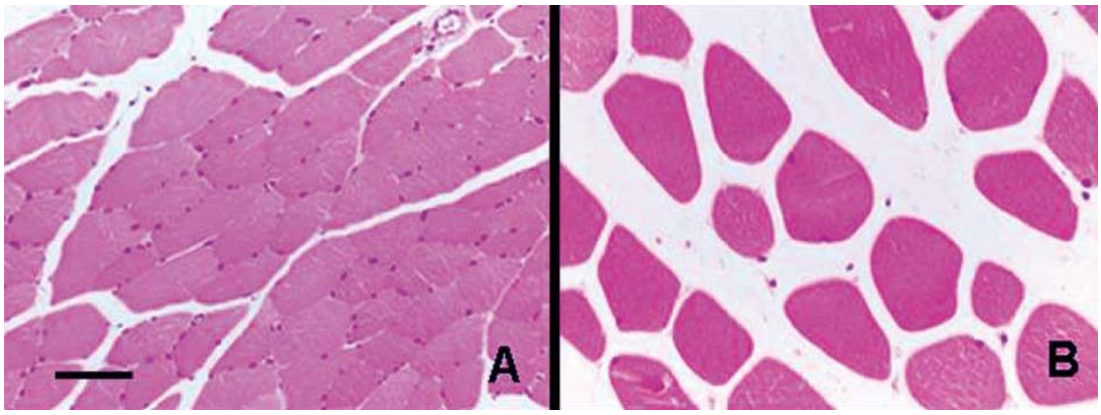


Figure 53 – Hematoxylin-eosin (H&E) stained gastrocnemius muscle from Asano *et al*'s model A) normal muscle B) ischaemic muscle (218)

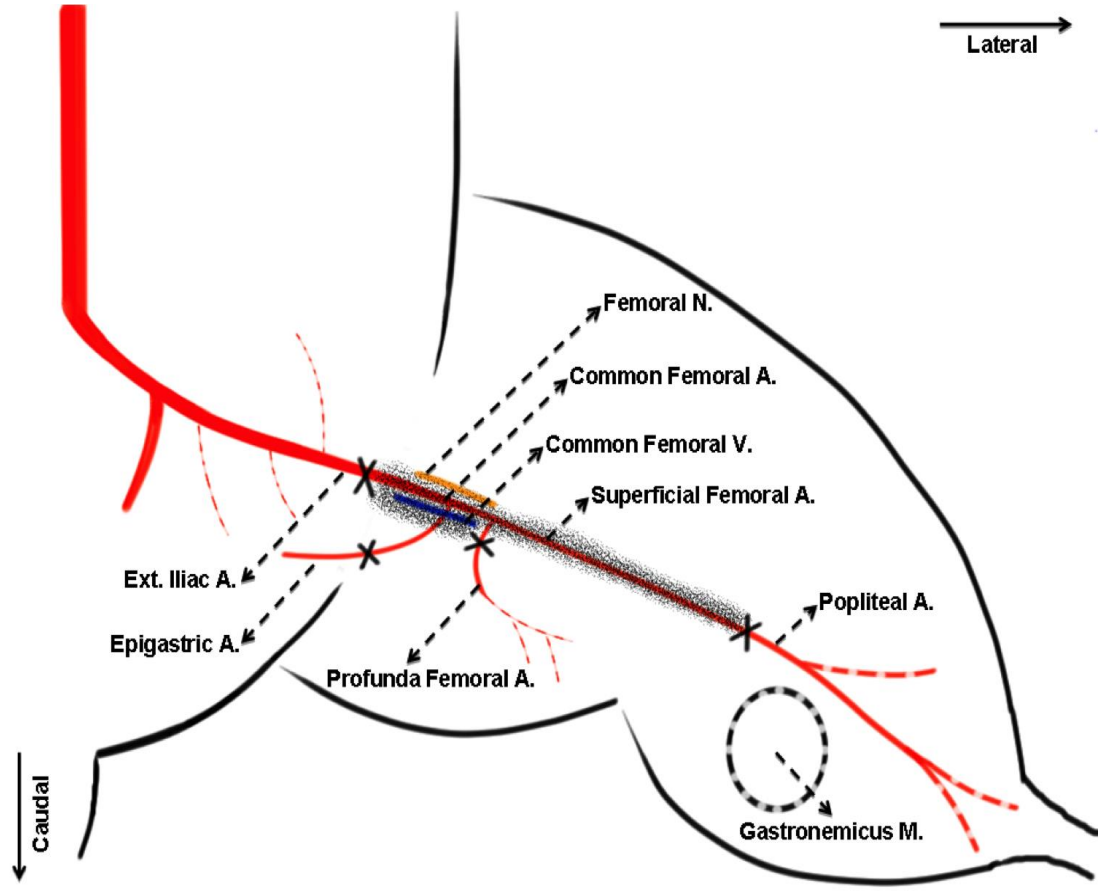


Figure 54 – Illustrating the mouse model of hind-limb ischaemia used in this study where excising the common and superficial femoral artery were excised and the side branches were ligated

Figure 55 - Operating theatres in animal lab at Royal Free campus, UCL, London.

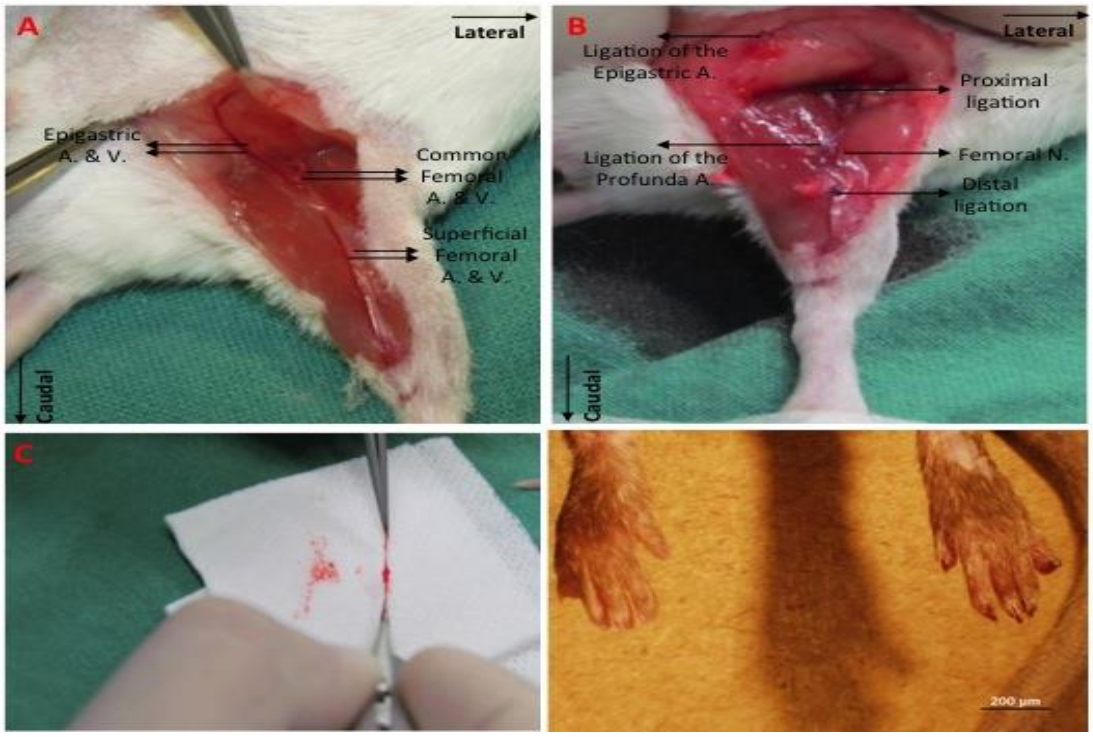


Figure 56 – A) Demonstrating the anatomy of the left mouse hind limb, supine position. Extensive dissection of the skin and fascia is for the purpose of demonstration. Common femoral artery appears below the inguinal ligament and gives off two main branches of epigastric and profunda before it forms the superficial femoral artery. B) Demonstrating the anatomy of the left mouse hind-limb after ligation of the femoral artery at the proximal and distal sites before the excision of the femoral artery. C) Showing the excised artery. D) Day 1 post operation with toe nail necrosis.

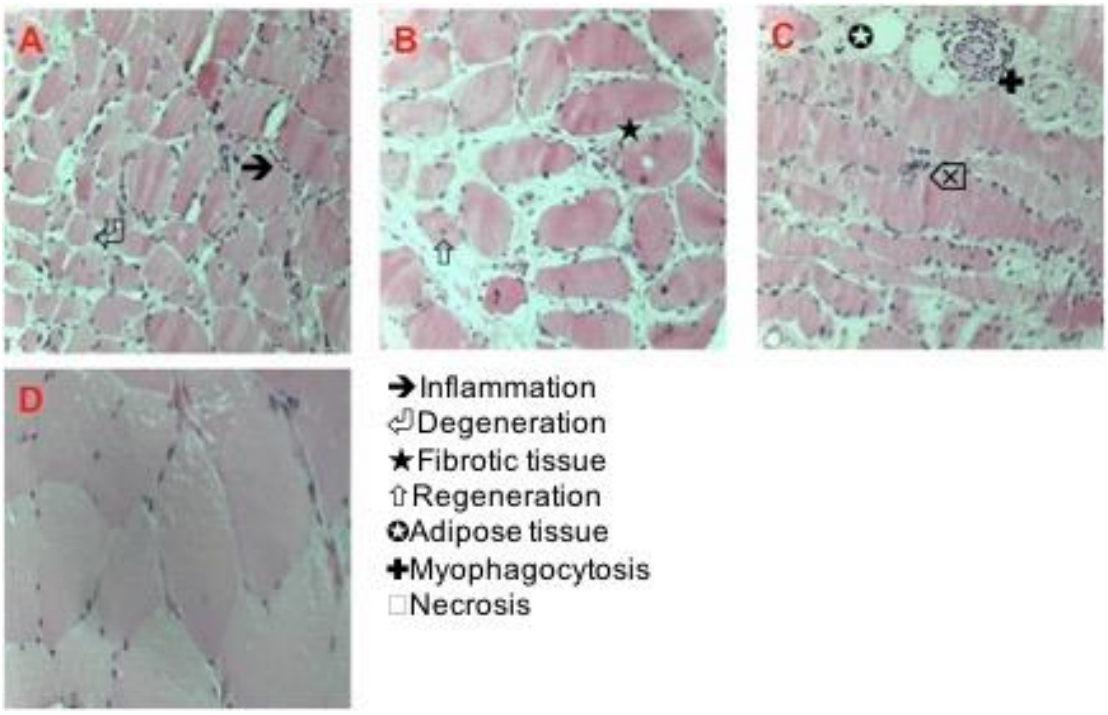


Figure 57 – H&E staining of gastrocnemius muscle from a patient with CLI demonstrating A) Inflammatory cell infiltration and degeneration and atrophic fibres, B) Fibrosis and regeneration with central nuclei C) Adipose tissue replacement, myophagocytosis and necrosis (nuclear clumps); D) H&E staining of gastrocnemius muscle from a patient with no PAD demonstrating homogenous, polygonal shaped muscle fibres with peripherally located nuclei.

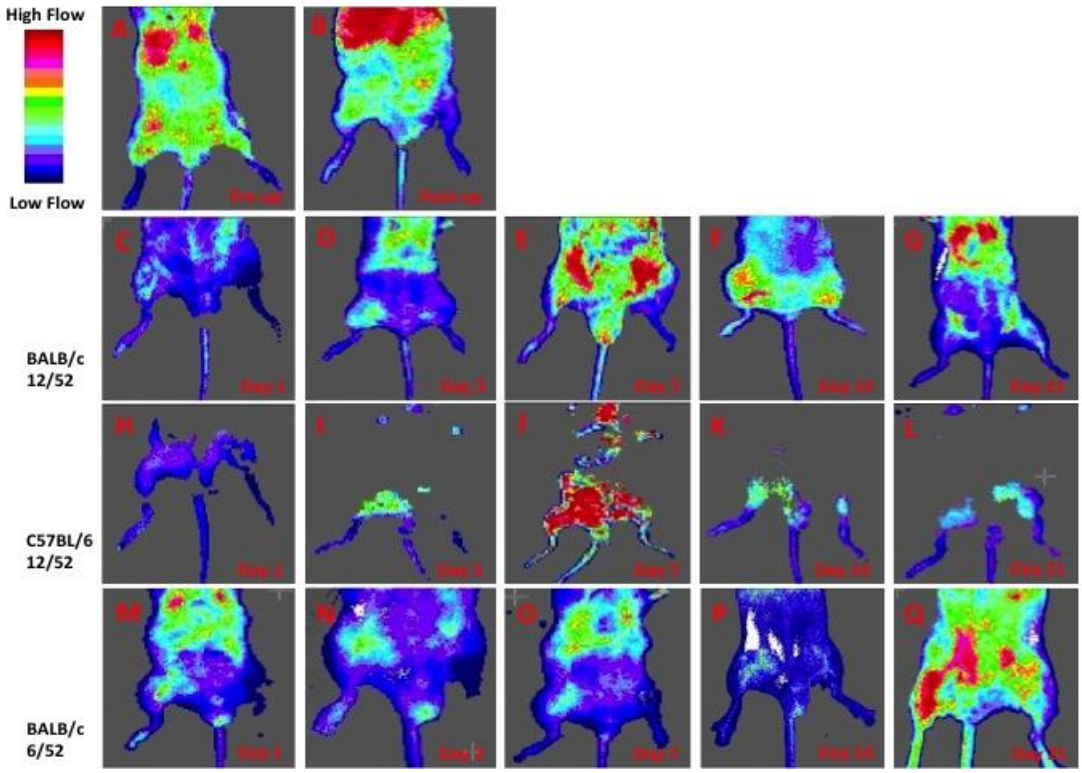


Figure 58 - Laser Doppler imaging perfusion measurements of the mice positioned supine under the scan head on a low temperature heating pad. Serial colour-coded perfusion images A) before and B) immediately after surgery. 12 weeks old male BALB/c mouse C) Day 1, D) Day 3, E) Day7, F) Day14, G) Day 21 after surgery. 12 weeks old male C57BL/6 mouse H) Day 1, I) Day 3, J) Day 7, K) Day 14, L) Day 21 after surgery. 6 weeks old male BALB/c mouse M) Day 1, N) Day 3, O) Day 7, P) Day 14, Q) Day 21 post surgery.

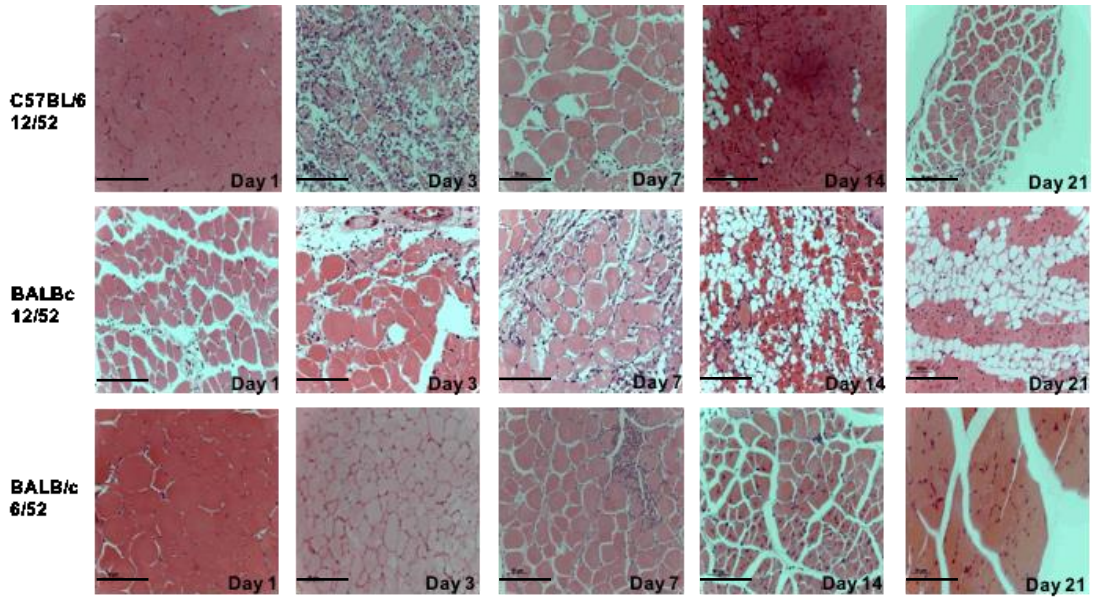


Figure 59 – Gastrocnemius muscle biopsy (Ischaemic limb); day 1, 3, 7, 14, 21 in C57BL/6-12 weeks old, BALBc-12 weeks old and BALBc-6 weeks old (scale bar = 10-50μm)

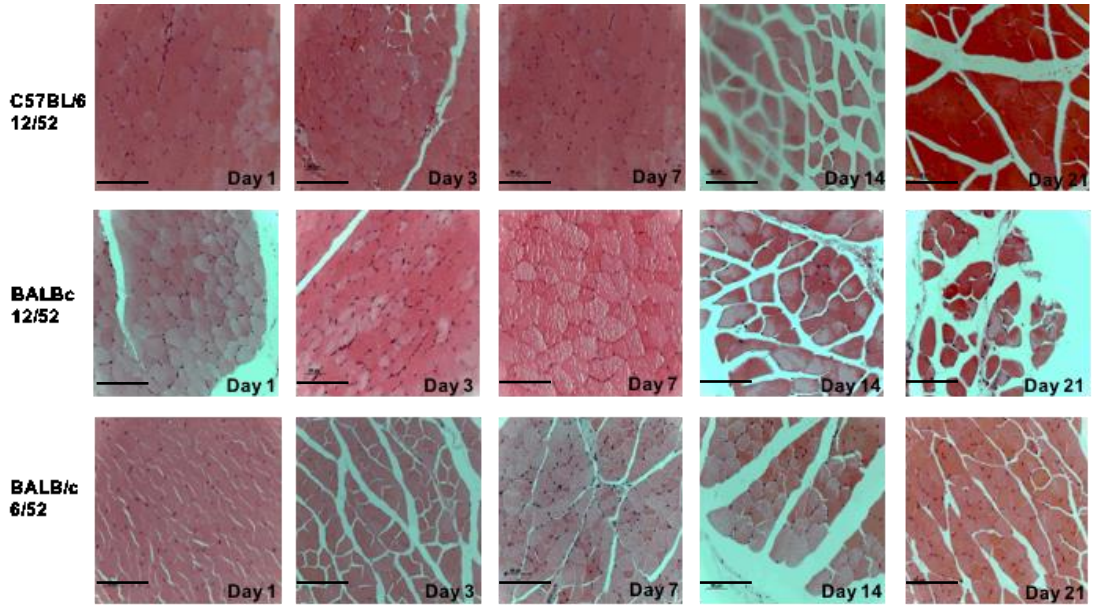


Figure 60 – Gastrocnemius muscle biopsy (non-Ischaemic limb); day 1, 3, 7, 14, 21 in C57BL/6-12 weeks old, BALBc-12 weeks old and BALBc-6 weeks old (scale bar = 10-50μm)

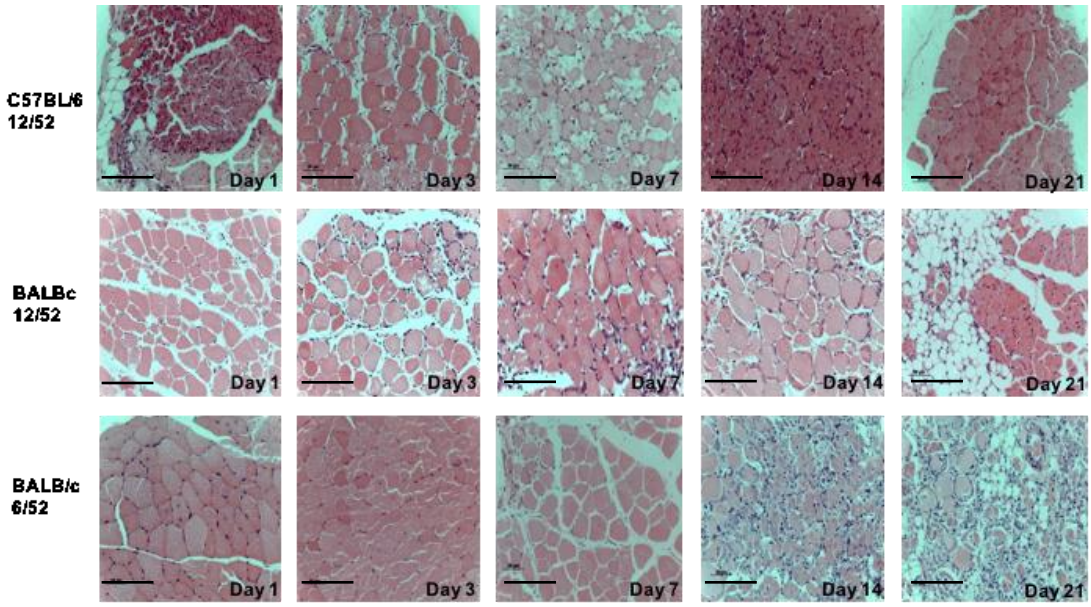


Figure 61 - Tibialis anterior muscle biopsy (ischaemic limb); day 1, 3, 7, 14, 21 in C57BL/6-12 weeks old, BALBc-12 weeks old and BALBc-6 weeks old (scale bar = 10-50µm)

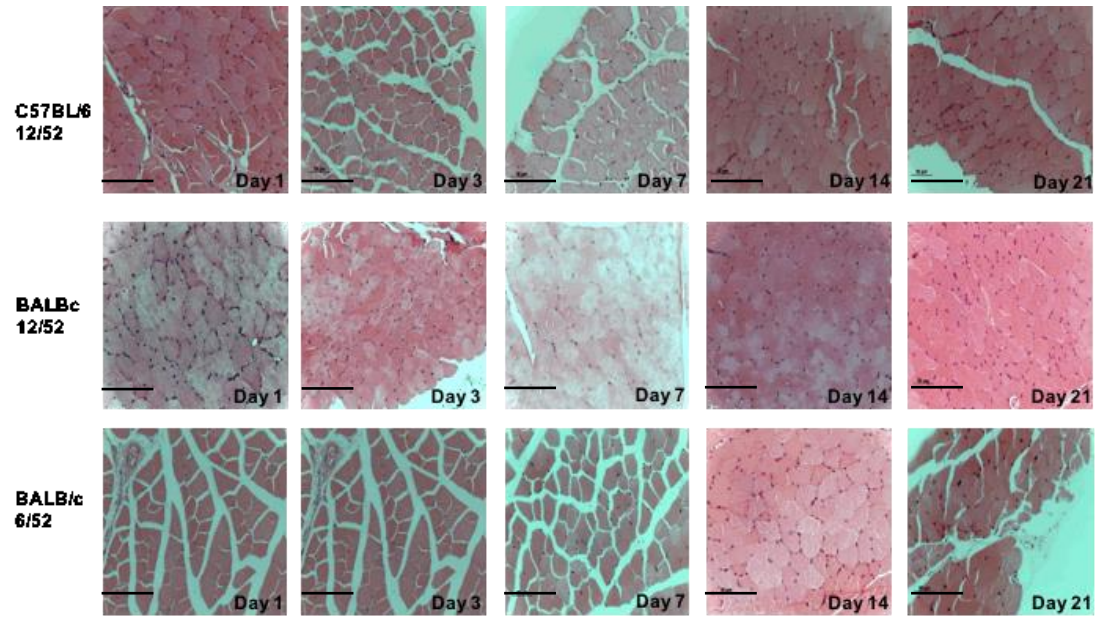


Figure 62- Tibialis anterior muscle biopsy (non-ischaemic limb); day 1, 3, 7, 14, 21 in C57BL/6-12 weeks old, BALBc-12 weeks old and BALBc-6 weeks old (scale bar = 10-50µm)

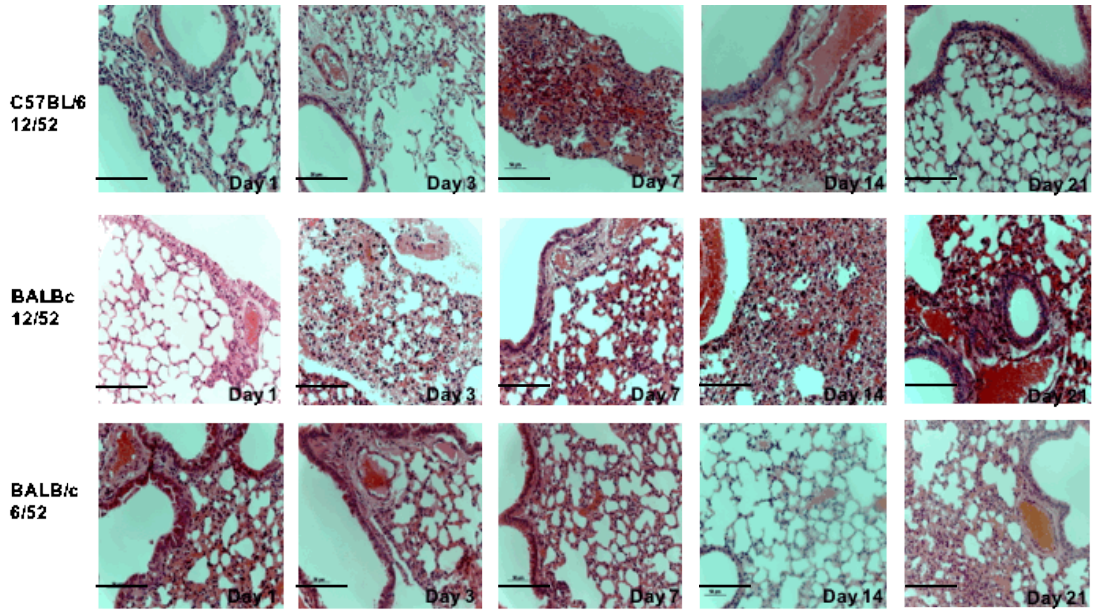


Figure 63 – H&E staining of lung biopsies; day 1, 3, 7, 14, 21 in C57BL/6-12 weeks old, BALBc-12 weeks old and BALBc-6 weeks old (scale bar = 10-50µm)

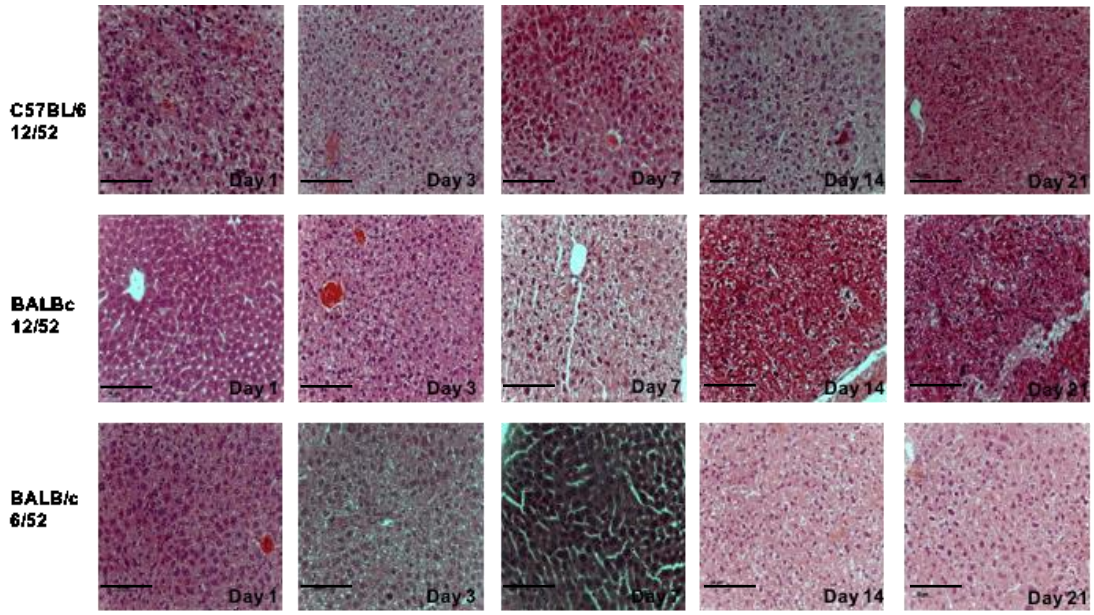


Figure 64 – H&E staining of liver biopsies; day 1, 3, 7, 14, 21 in C57BL/6-12 weeks old, BALBc-12 weeks old and BALBc-6 weeks old (scale bar = 10-50µm)

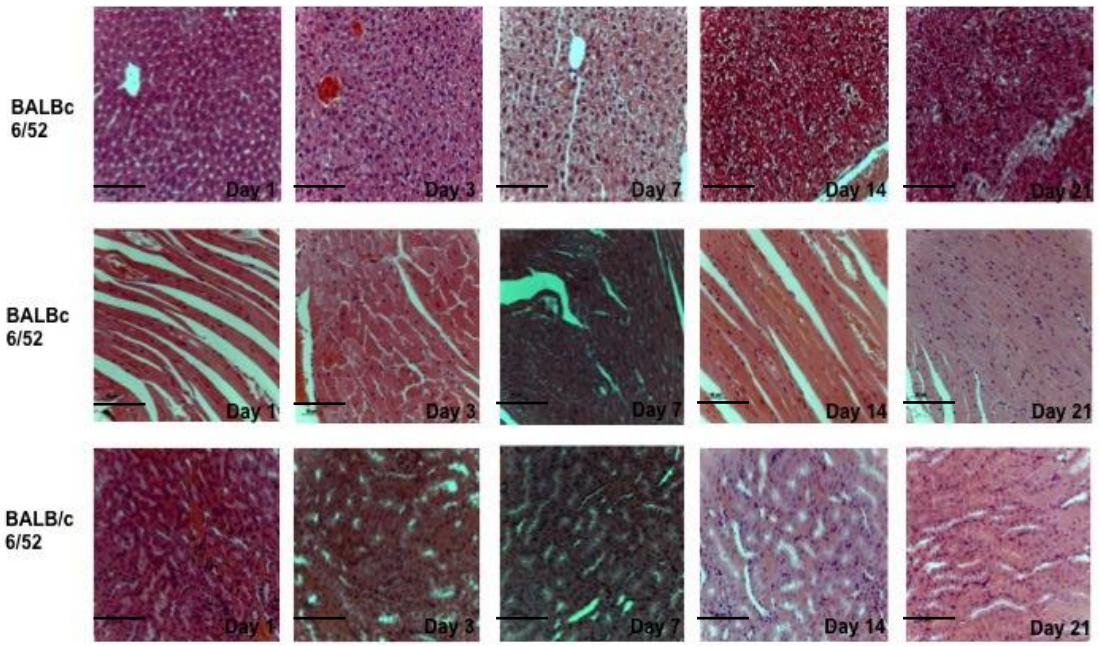


Figure 65 – H&E staining of liver, heart and kidney biopsies; day 1, 3, 7, 14, 21 in BALB/c-6 weeks old (scale bar = 10-50µm)

CHAPTER 6: THERAPUTIC ROLE OF TLR4 INHIBITION IN MOUSE MODEL OF HIND-LIMB ISCHAEMIA

6. 1 Introduction

6. 2 Aims

6. 3 Experimental design

6. 4 Results

6. 5 Discussion

6. 1. Introduction

TLR antagonists and inhibitors are currently in various stages of development from pre-clinical evaluations to clinical trials. TLR4 antagonists are being developed and tested with the objective to inhibit acute exacerbation of organ damaging immune responses. The TLR4 antagonist Eritoran, showed positive results in the treatment of critically ill septic patients in a randomized, double-blind, multicentre phase II clinical trial (NCT00046072), however in phase III studies Eritoran did not perform better than existing treatments for the treatment of sepsis. Another TLR4 antagonist, TAK242, has also progressed to two phase III clinical trials (NCT00143611 and NCT00633477) (234).

Although TLR4 has a pathogen detection role, as previously discussed, it can act as a monitoring receptor in the detection of tissue injury. Endogenous ligands such as HSPs and HMBG1 are up-regulated during ischaemia-induced tissue damage. The release of TLR4 endogenous ligands is associated with TLR4 activation and the corresponding inflammatory response can lead to excessive tissue damage. Thus, pharmacological inhibition of TLR4 can be of potential therapeutic benefit.

Zhao *et al*. showed that TLR4 has a detrimental role in renal transplant ischaemic reperfusion injury as it prompts maladaptive immune response that results in excess tissue damage. The authors argued that TLR4 inhibition could serve as a target for pharmacological agents directed toward optimizing renal graft survival (235).

Nace *et al.* investigated the role of TLR4 in liver ischaemia using TLR4 knock out mice. The exposure of TLR4 knock out mice to liver ischaemia was associated with reduced phosphorylation of NFkB, JNK and P38, as compared to wild type mice. The endogenous inhibition of TLR4 was associated with less inflammatory response in liver ischaemia (236).

Suzuki *et al.* examined the role of pharmacological inhibition of TLR4 in neuronal death in transient brain ischaemia. The exogenous inhibition of TLR4 in a mouse model of transient brain ischaemia, just before reperfusion, was associated with less cerebral ischemia-induced oxidative stress and reduced production of the reactive oxygen species (237). The effect of exogenous TLR4 inhibition has also been found to improve neurological function in a mouse model of middle cerebral artery occlusion (238).

Shimamoto *et al*. investigated the role of TLR4 inhibition in attenuation of myocardial ischaemia reperfusion injury. Exogenous inhibition of TLR4 in a mouse model of myocardial ischaemia was associated with reduced ischaemia-induced injury and markers of inflammatory response. This was evidenced by reduced infarct size, TNF**α** and IL-6 levels (48).

TLR4-induced inflammatory response has been shown to encourage angiogenesis and collateral artery formation in various ischaemic reperfusion models suggesting the dual role of TLR4 in inflammation and angiogenesis. However, it has been reasoned that fine tuning of TLR4 and its associated signalling pathway can eliminate the undesired effect of TLR4 inhibition on collateral artery formation (239).

Borne *et al.* studied the effect of systemic inhibition of TLR4 on perfusion recovery in a mouse model for angiogenesis. They found that inhibition of TLR4 by TAK-242 in a mouse model of hind-limb ischaemia did not negatively influence perfusion recovery following the ischemia, despite its potential inhibitory effects on angiogenesis (240).

If TLR4 inhibition is able to reduce ischaemia-induced skeletal muscle damage effectively, targeting the TLR4 pathway could provide an additional / alternative therapeutic benefit in PAD.

6. 2. Aims

In vitro TLR inhibition reduced apoptotic changes in human myotubes cultured in ischaemic conditions. Here, the aim was to investigate the therapeutic potential of modulating TLR4 signalling *in vivo*.

Objectives:

The objective was:

- To assess the effect of TLR4 inhibition on skeletal muscle damage in a mouse model of hind-limb ischaemia

Hypothesis:

It was hypothesized that:

- Hind-limb ischaemia will result in TLR4 upregulation and activation whilst endogenous deletion and exogenous inhibition of TLR4 will attenuate tissue damage following hind-limb ischaemia.

6. 3. Experimental design

Hind-limb ischaemia was induced by excision of the common and superficial femoral arteries and ligation of the side branches [Figure 66] in male C57BL/6 mice of 12 weeks of age, as described in chapter 5. The animals were split into three groups:

- Group 1: Endogenously deleted TLR4 (TLR4-/-); n=18
- Group 2: Exogenously inhibited TLR4 (LPS-RS); n=18
- Group 3: Vehicle; n=18

Nine animals per group were sham operated [Figure 67]. Mice in group 1 were kindly gifted by Dr Nathan Davies, Institute for Liver & Digestive Health, UCL Medical School, Royal Free Campus, London (241). These animals with

TLR4lps-del spontaneous mutation had a 7-kb deletion in the TLR4 gene (Jackson Laboratory, United States). Two breeding pairs of TLR4-knockout mice were purchased from the Jackson Laboratory and bred in house. Mice in groups 2 and 3 were purchased from the UCL Biological Services (Royal Free Campus).

Mice in group 2 were treated by intraperitoneal administration of LPS-RS (1µg per mouse (40µg/kg) in saline, twice a week; first injection at the time of induction for surgery; InvivoGen). This dose of LPS-RS was reported to be effective in blocking TLR4 in previous studies (242-244). Laser Doppler (Moor FLPI; Moor Instruments Ltd) was used to measure changes in perfusion. The overall well-being of animals was checked daily with specific assessment of function performed on post surgical day 3, 7 and 21. Tissue necrosis, limping and muscle flexion were recorded to calculate the post-operative functional scores. The Tarlov and Modified Ischaemia scoring systems were used for this purpose (243) [Table 9].

Animals were sacrificed (n=6 per ischaemic group and n=3 per sham group at each time point) and hind-limb tissue and serum samples were collected at day 3, 7 and 21. Blood samples were taken via cardiac puncture at the time of sacrificing the animals.

TLR4 mRNA expression was examined by RT-PCR, as previously described in chapter 2. 5’ sense and 3’ antisense TLR4 primers, provided with positive control double stranded DNA were used for real-time PCR (rtp-mtlr4; InvivoGen). Systemic levels of IL-6 & TNFα were examined by ELISA (Mouse IL6, M6000B, R&D Systems; Mouse TNFα, MTA00B, R&D Systems). Expression of cleaved caspase-3 in the ischaemic skeletal muscle was examined by Fluorescent IHC-P (Mouse Cleaved Caspase-3, rabbit antibody, 9661; Cell Signalling; Alexa Fluor® 594 Chicken Anti-Rabbit IgG, A-21442, Life Technologies). Inflammatory damage was assessed by H&E staining [Figure 68]. Inflammatory cell quantification was carried out using Image J computer software (ImageJ 1.4s, National Institutes of Health, USA).

Further, blood gas analysis was carried out at each time point to measure the following: pO_2, pH, pCO_2, haemoglobin (Hb), lactate, Sodium (Na), Potassium (K), glucose and SO_2.

ARRIVE guidelines were followed during the design and analysis of the in vivo experiments (245).

Score	Tarlov scoring
0	No Movement
1	Barely perceptible movement, non-weight-bearing
2	Frequent movement, non-weight-bearing
3	Supports weight, partial weight-bearing
4	Walks with mild deficit
5	Normal but slow walking
6	Full and fast walking

Score	Modified ischaemia scoring
0	Auto-amputation of leg
1	Leg necrosis
2	Foot necrosis
3	Discoloration of > 2 toes
4	Discoloration of 1 toe
5	Discoloration of > 2 nails
6	Discoloration of 1 nail
7	No necrosis

Table 9 – Recognised functional and ischaemia scoring based on mobility/ muscle function and degrees of tissue ischaemia (246)

6. 4. Results

There was upregulation of TLR4 mRNA in gastrocnemius muscle of mice who underwent hind-limb ischaemia compared with that of sham operated mice (group 3) at day 3 (ΔCt: median 9, range 2.7-16 *vs.* median 2, range 1-2.6) and day 21 (ΔCt: median 10, range 9-11 *vs.* median 1, range 1-1) [Figure 69]. Analysis of the laser Doppler images showed significant reduction in perfusion in the operated hind-limb at day 3 post surgery in all groups (perfusion unit rate: vehicle (n=6), median 0.38; TLR4 -/- (n=6), median 0.2; LPS-RS (n=6), median 0.41), with the reduction being more significant in the TLR4-/- group when compared to vehicle and LPS-RS groups at this time point ($P<0.05$, Mann Whitney test) [Figure 70, Figure 72]. The perfusion in the sham operated groups showed no significant differences between the groups at all time points [Figure 71]. Slower blood flow recovery was observed in vehicle group in comparison to TLR4-/- and LPS-RS groups at day 3 (perfusion unit rate: vehicle (n=6), median 0.38; TLR4 -/- (n=6), median 0.2; LPS-RS (n=6), median 0.41), day 7 (perfusion unit rate: vehicle (n=6), median 0.51; TLR4 -/- (n=6), median 0.79; LPS-RS (n=6), median 0.61), and day 21 (perfusion unit rate: vehicle (n=6), median 0.84; TLR4 -/- (n=6), median 1.1; LPS-RS (n=6), median 0.90) ($P<0.05$, Kruskal Wallis test) [Figure 72].

The functional status of the mice was assessed at day 3, 7 and 21 post surgery [Table 10] [Figure 73].

Day 3	Tarlov score (median)	Modified ischaemia score (median)
Vehicle (n=6)	1.0	3.1
TLR4 -/- (n=6)	3.0	3.6
LPS-RS (n=6)	1.1	3.5

Day 7	Tarlov score (median)	Modified ischaemia score (median)
Vehicle (n=6)	3.9	5.5
TLR4 -/- (n=6)	5.2	5.5
LPS-RS (n=6)	4.9	5.5

Day 21	Tarlov score (median)	Modified ischaemia score (median)
Vehicle (n=6)	4.9	5.4
TLR4 -/- (n=6)	5.9	7
LPS-RS (n=6)	5.8	6.9

Table 10 - The functional status of the mice was assessed at day 3, 7 and 21 post surgery.

All mice showed low functional performance post surgery with gradual improvement by day 21. Mice in TLR4-/- and LPS-RS groups restored their functional status to the near base line by day 21, whereas the vehicle group had lower performance even at day 21 ($P<0.05$; Kruskal Wallis test) [Figure 73, Figure 74].

The blood gas analysis of blood samples taken via cardiac puncture at the time of sacrificing the animals, showed no significant differences between the ischaemic and sham operated mice in both vehicle and TLR4 -/- groups when the following parameters were quantified: pH, pO_2, pCO_2, Hb, lactate, Na, K and SO_2. The random blood glucose levels in ischaemic and sham operated TLR4 -/- groups were significantly higher compared to ischaemic and sham operated vehicle groups at day -1, 3, 7 and 21 ($P<0.05$; Kruskal Wallis test) [Figure 75, Figure 76].

The ELISA quantification of IL-6 showed significantly increased levels in the vehicle group compared to TLR4-/- and LPS-RS groups at day 3, 7 and 21 post surgery ($P<0.05$; Kruskal Wallis test) [Table 11] [Figure 77].

Day 3	IL-6 (pg/ml)
Vehicle (n=6)	20
TLR4 -/- (n=6)	9
LPS-RS (n=6)	11

Day 7	IL-6 (pg/ml)
Vehicle (n=6)	26
TLR4 -/- (n=6)	11
LPS-RS (n=6)	20

Day 21	IL-6 (pg/ml)
Vehicle (n=6)	35
TLR4 -/- (n=6)	20
LPS-RS (n=6)	22

Table 11 - ELISA quantification of IL-6 at day 3, 7 and 21 post surgery

Similarly, ELISA quantification of TNFα showed significantly increased levels in the vehicle group in compare to TLR4-/- and LPS-RS groups at day 3, 7, 21 post surgery ($P<0.05$; Kruskal Wallis test) [Table 12] [Figure 77].

Day 3	TNFα (pg/ml)
Vehicle (n=6)	280
TLR4 -/- (n=6)	30
LPS-RS (n=6)	125

Day 7	TNFα (pg/ml)
Vehicle (n=6)	310
TLR4 -/- (n=6)	90
LPS-RS (n=6)	150

Day 21	TNFα (pg/ml)
Vehicle (n=6)	360
TLR4 -/- (n=6)	119
LPS-RS (n=6)	160

Table 12 - ELISA quantification of TNFα at day 3, 7 and 21 post surgery

TLR4-/- mice and mice given LPS-RS, demonstrated diminished apoptosis in ischaemic skeletal muscle following hind-limb ischaemia as compared to vehicle mice at day 21 [Figure 78]. Fluorescent immunohistochemical staining for cleaved-caspase 3 showed less expression of this protein in TLR4 inhibited groups at day 21.

Inflammatory cell quantification in H&E stained paraffin embedded ischaemic gastrocnemius muscle sections post surgery showed less inflammatory cell infiltration in both TLR4 -/- and LPS-RS groups when compared to vehicle group at each time point ($P<0.05$; Kruskal Wallis test) [Figure 79]. The median percentages of inflammatory cell infiltration in vehicle, TLR4 -/- and LPS-RS groups were calculated as 19%, 7% and 10.5% respectively at day 3; 21%, 10% and 14% respectively at day 7, and 14%, 8% and 10.5 % respectively at day 21.

TLR4-/- mice and mice given LPS-RS demonstrated attenuated histological evidence of ischaemia-induced inflammation following hind-limb ischaemia as compared to vehicle mice at days 3, 7 and 21 [Figure 80, Figure 81].

The H&E examination of the hind-limb skeletal muscles in sham groups showed no obvious histological differences between vehicle, TLR4 -/- and LPS-RS groups at day 3, 7 and 21 [Figure 82, Figure 83].

6. 5. Discussion

In this chapter, the role of TLR4 inhibition *in vivo* was examined in a murine model of hind-limb ischaemia. TLR4 up-regulation occurred in ischaemic skeletal muscle (group 3) with increased TLR4 mRNA expression in ischaemic gastrocnemius muscle post surgery at day 3 and 21, however the sample size was small.

Tissue perfusion was significantly interrupted after the excision and ligation of the femoral artery and its side branches. This was followed by recovery of tissue perfusion in all experimental groups, however the mice with compromised TLR4 function showed faster recovery when compared to the vehicle group. Functional assessment of the mice showed reduced function in all three groups post surgery, however the functional performance was superior in TLR4 inhibited groups at day 21.

Blood gas analyses showed no significant differences in the measurements of pO_2, pH, SO_2 and Hb between the vehicle and TLR4-/- groups. These markers could potentially act as confounding factors by influencing the oxygen delivery equation. The random blood glucose levels were higher in TLR4-/- groups (both ischaemic and sham) even prior to the surgery; however, if hyperglycaemia was a potential confounding factor, it would be expected to perhaps worsen recovery in this group. These findings are consistent with previously published data suggesting that TLR4 controls the development of autoimmune diabetes in TLR4-/- mice by affecting the activity of regulatory T-cells (247).

In agreement with *in vitro* findings that showed TLR4 inhibition prior to

ischaemia in human myotubes was associated with reduced inflammatory cytokine production, endogenous deletion and exogenous inhibition of TLR4 *in vivo* were associated with reduced systemic IL6 and TNFα levels following hind-limb ischaemia. Further experiments showed that TLR4-/- mice and mice given LPS-RS, exhibited reduced ischaemia-induced apoptosis similar to that observed in *in vitro* experiments.

In addition, TLR4 inhibition was associated with diminished inflammatory cell infiltration in the ischaemic limb as compared to mice treated with vehicle at different time points. Endogenous deletion and exogenous inhibition of TLR4 were associated with reduced damage to the skeletal muscle after the ischaemic insult. These findings suggest that TLR4 plays a key role in ischaemia-induced skeletal muscle damage [Figure 84].

Limitations:

The upregulation of TLR4 mRNA in ischaemic skeletal muscle was only examined in limited number of experiments (n=2 per group at day 3 and 21 post surgery). Further, the potential increase in other TLRs, as compensatory increase, was not examined in these experiments. The other limitation is that the animal model does not include the other cardiovascular risk factors that CLI patient experience. Furthermore, the effect of concomitant medications such as statins and anti-platelets is not observed.

Furthermore, many findings in animal models are not translated into the clinical setting, such as therapeutic angiogenesis that has shown more impressive results in preclinical studies than in clinical trials. This might be due to more complex systems along with more advanced diseases in human. Finally, the experimental drugs in animals undergo different pharmacokinetics and pharmacodynamics.

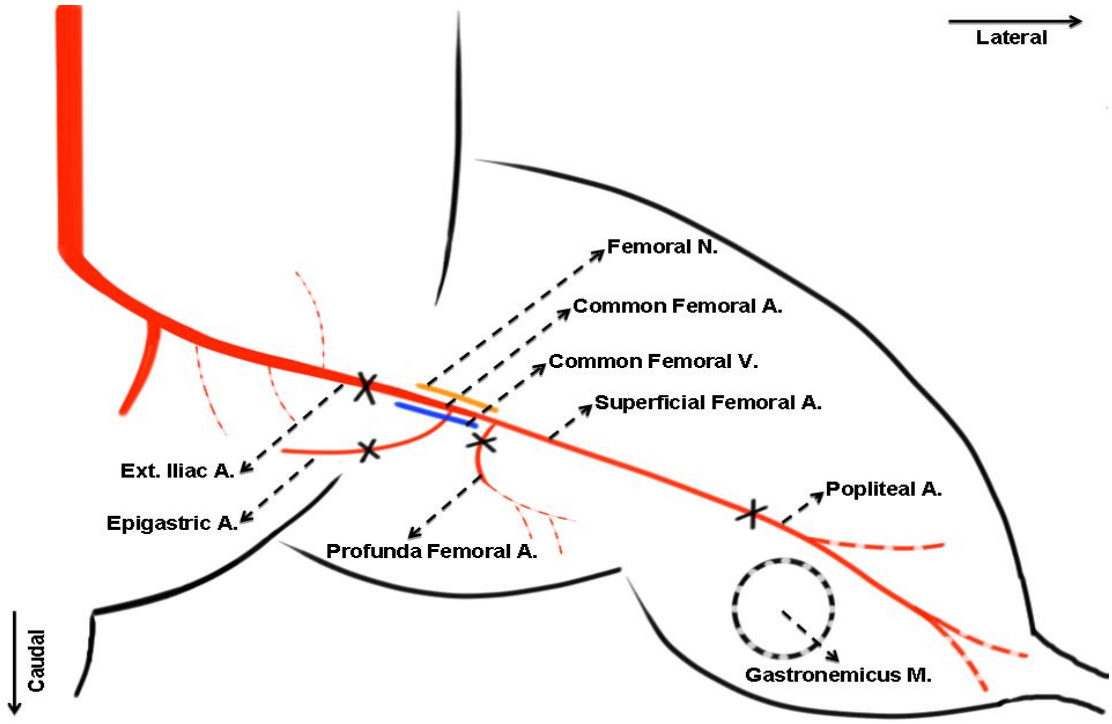


Figure 66 - The arterial tree was excised proximally above the inguinal ligament, and distally at the level of popliteal artery. The side branches were ligated at the points shown with X

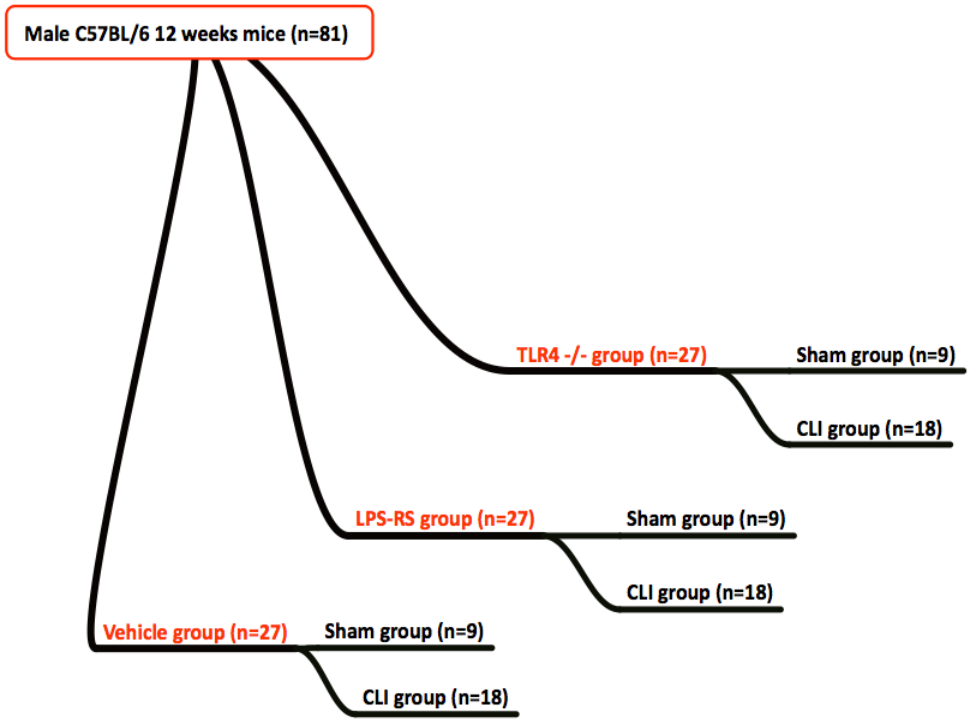


Figure 67 – *In vivo* experimental design and group allocations

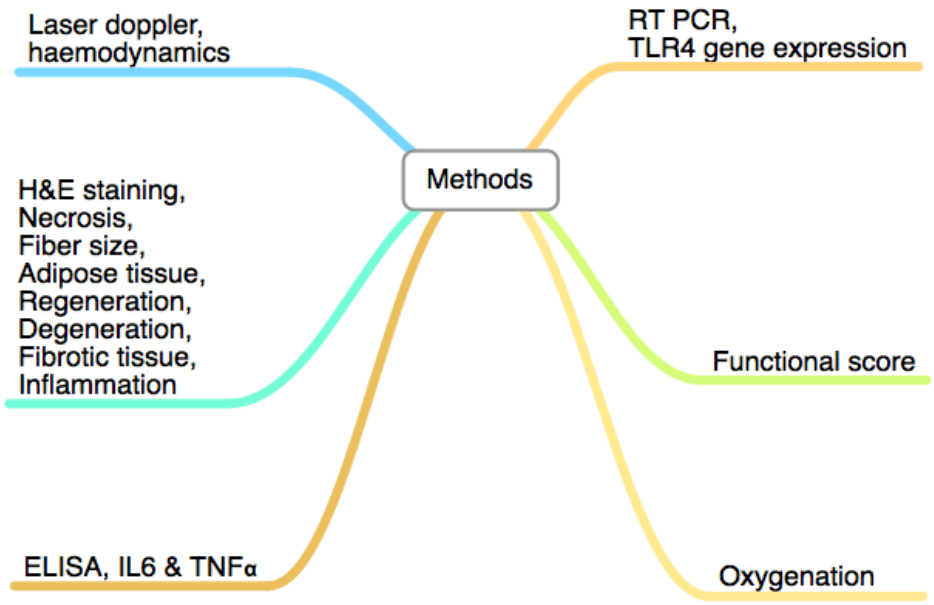


Figure 68 – *In vivo* experimental design

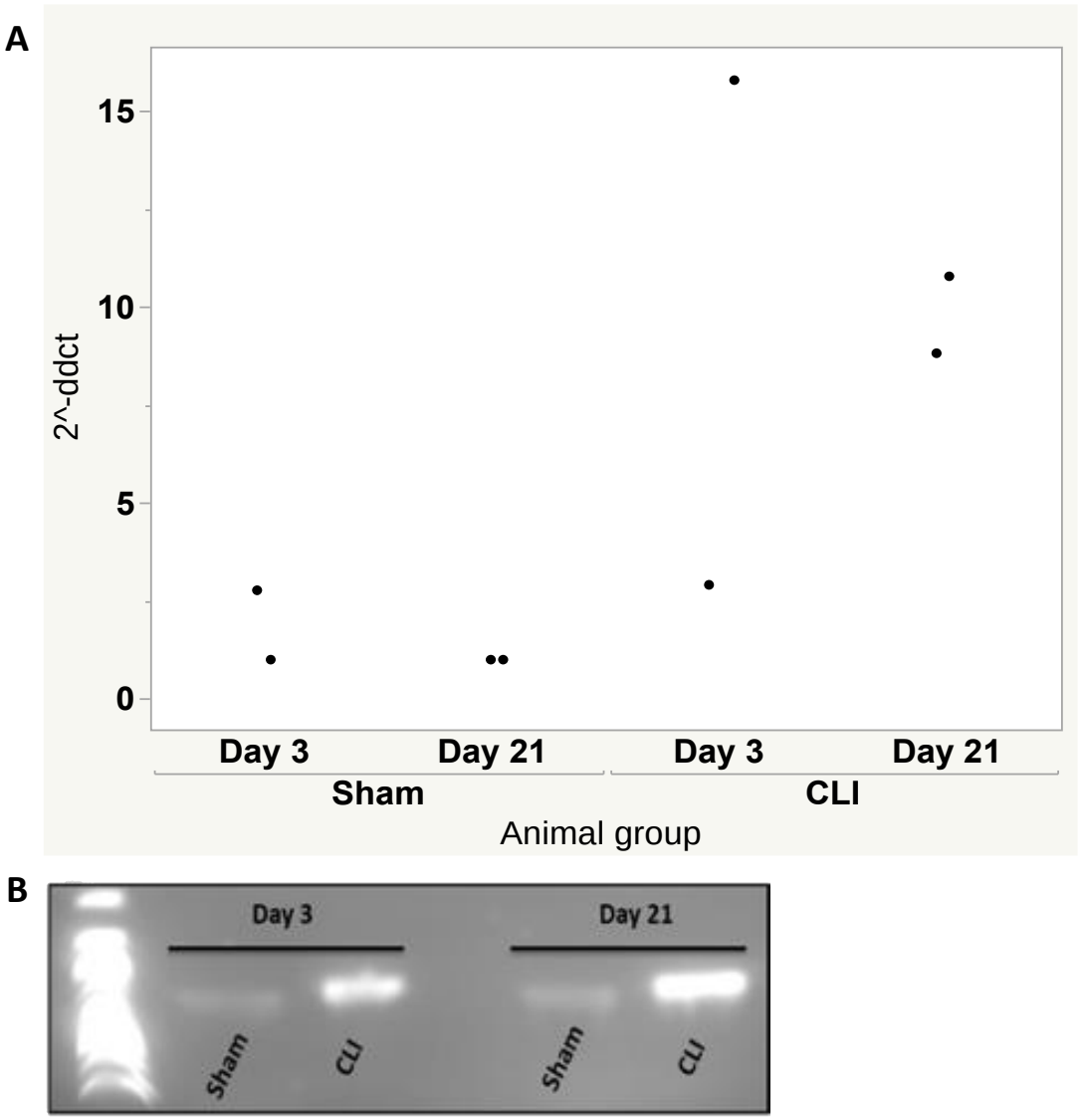


Figure 69 - RT-PCR quantification of TLR4 in mouse gastrocnemius muscle. Data has been normalized to endogenous housekeeping gene (tubulin). Vehicle group: CLI (n=2), Sham (n=2) on day 3 and 21 post operation. A) Fold expression of TLR4 across the groups at different time points (ΔCt analysis) B) Electrophoresis of the PCR products on 2% agarose gel

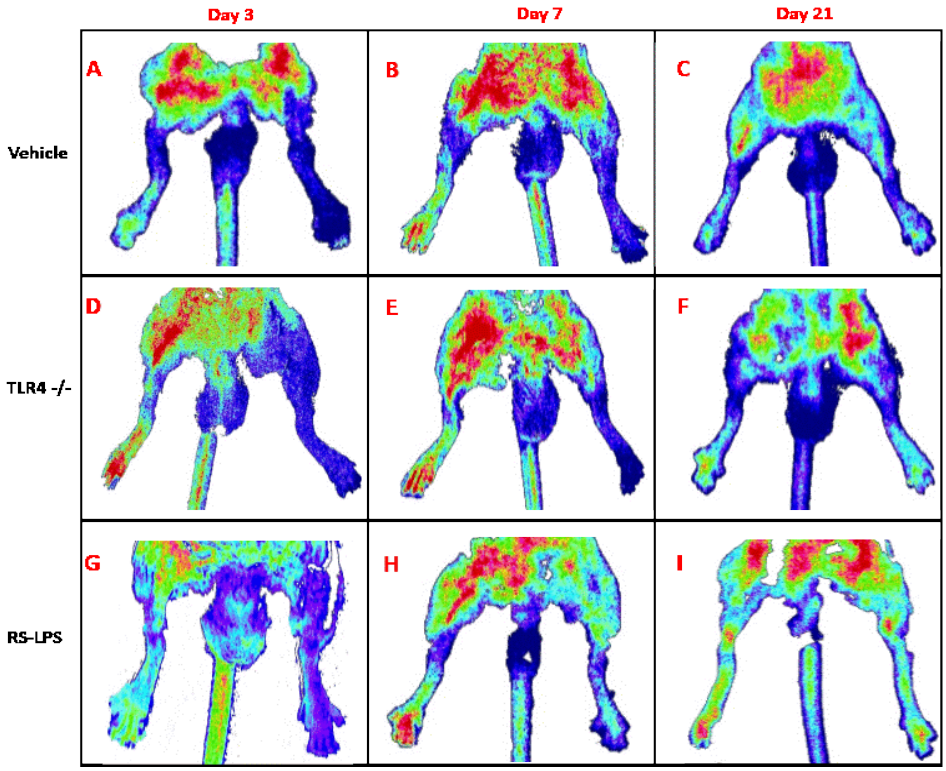


Figure 70 – Ischaemic groups - Representative Laser Doppler perfusion images obtained with mice positioned supine under the scan head on a low temperature heating pad. Serial colour-coded perfusion images A), B) & C) 12 weeks old C57BL/6, post operation on day 3, 7 & 21 respectively. D), E) & F) 12 weeks old C57BL/6 with genetically deleted TLR4, post operation on day 3, 7 & 21 respectively. G), H) & I) 12 weeks old C57BL/6 treated with exogenous TLR4 inhibition, post operation on day 3, 7 & 21 respectively.

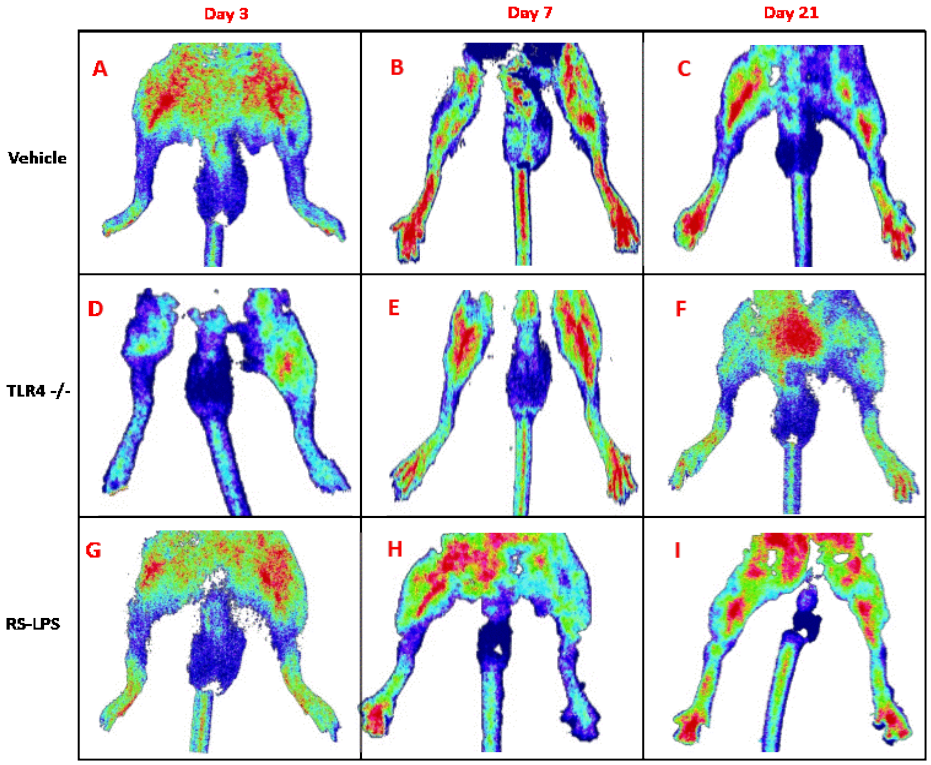


Figure 71 - Representative Laser Doppler perfusion images of mice positioned supine under the scan head on a low temperature heating pad. Serial colour-coded perfusion images A), B) & C) 12 weeks old C57BL/6, post Sham operation on day 3, 7 & 21 respectively. D), E) & F) 12 weeks old C57BL/6 with genetically deleted TLR4, post sham operation on day 3, 7 & 21 respectively. G), H) & I) 12 weeks old C67BL/6 treated with exogenous TLR4 inhibition, post sham operation on day 3, 7 & 21 respectively.

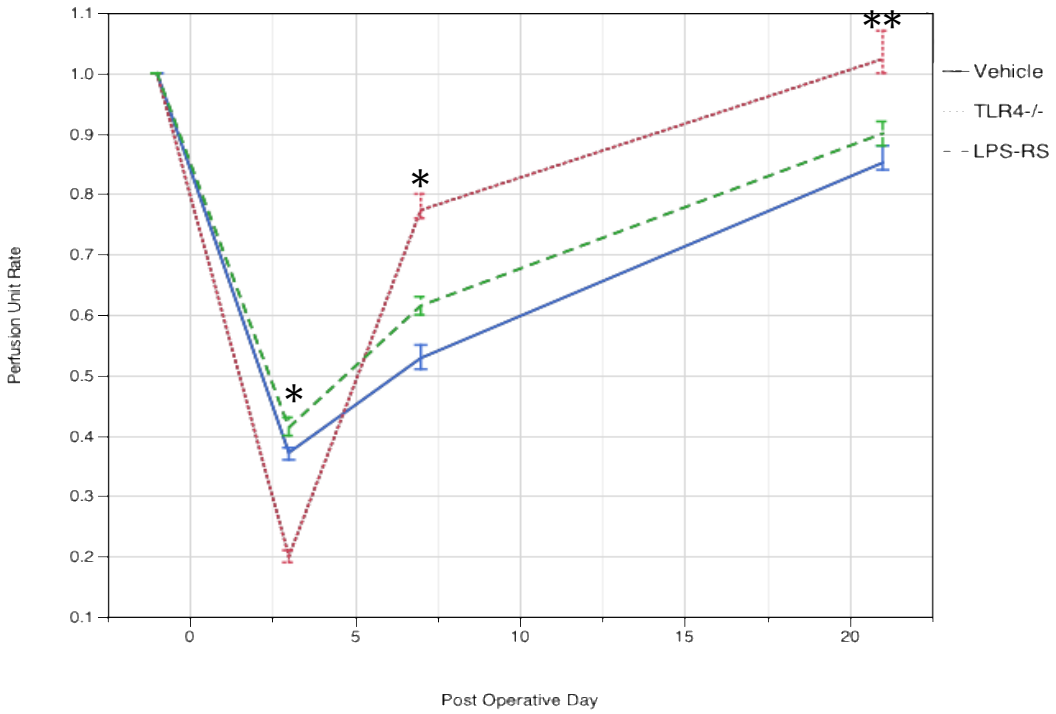


Figure 72 - Perfusion recovery post surgery. The ischaemic / non-ischaemic lower limb perfusion ratio showed significant difference on day 3, 7 and 21 between TLR4-/- group and Vehicle / LPS-RS groups (*P<0.001 at day 3 and 7; **P=001 at day 21; Kruskal Wallis test). The difference between each group was also significant (P<0.005; Mann Whitney test) on day 3, 7 and 21.

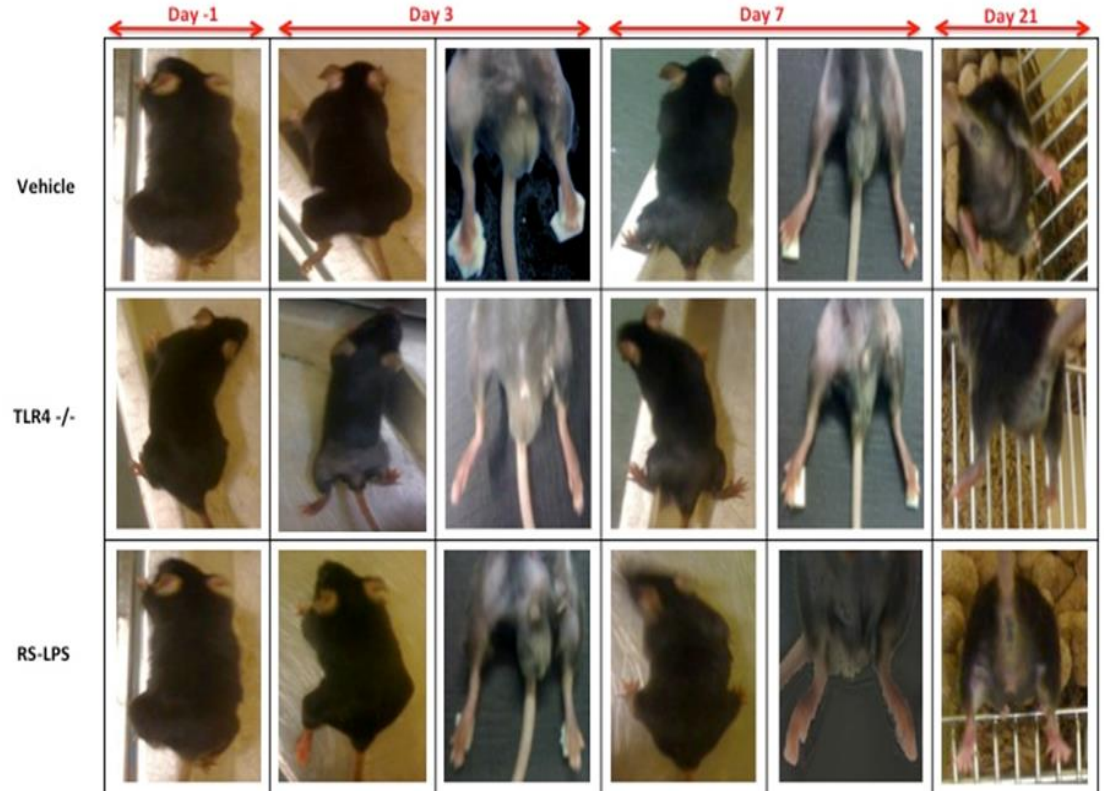


Figure 73 - Representative photographs of mice from all three groups that underwent hind-limb ischaemia at day -1, 3, 7 and 21. Tissue necrosis, limping and muscle flexion were recorded to calculate the functional and ischaemia scores post operation.

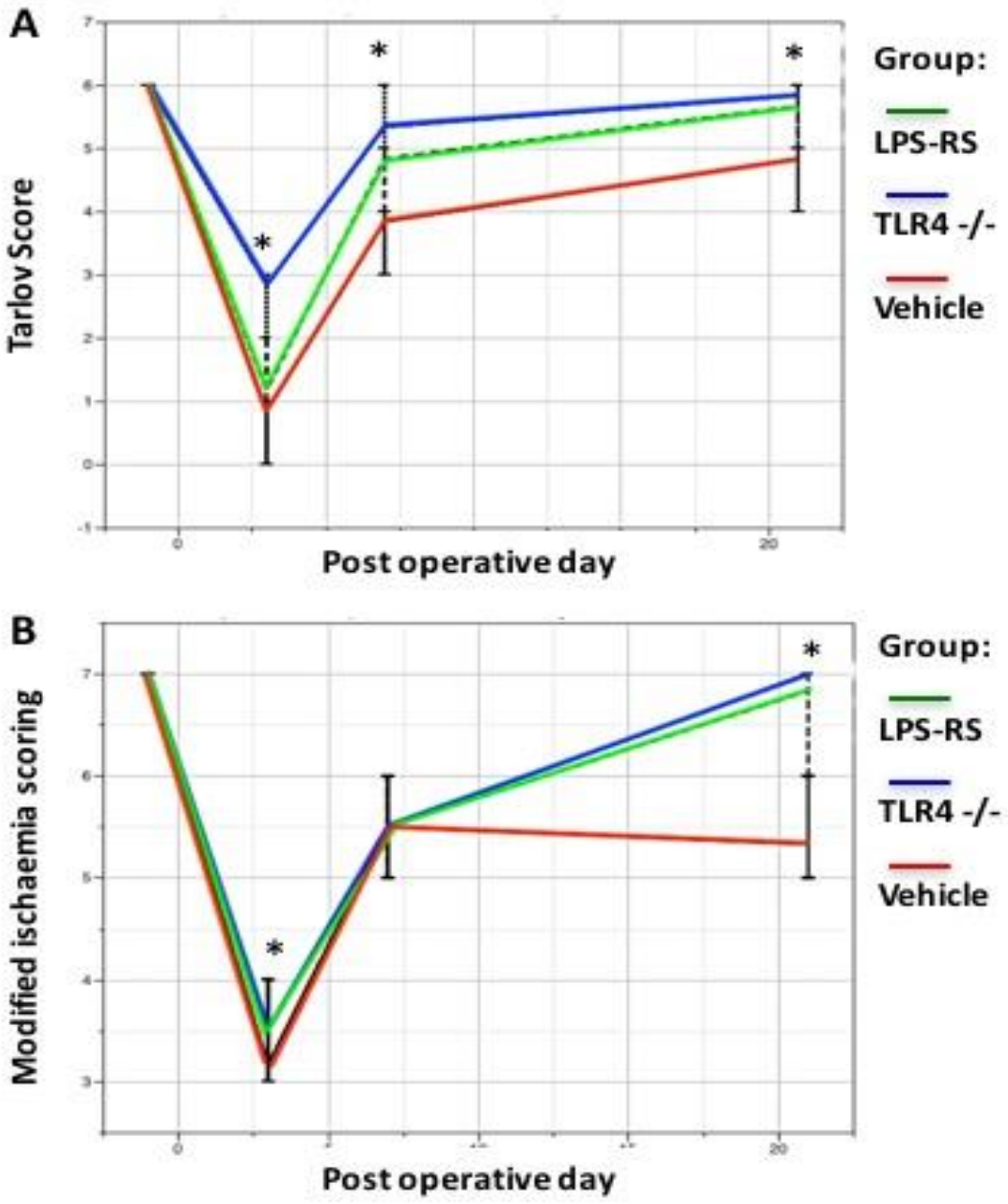


Figure 74 - Functional and ischaemia scoring of the mice in all groups showed faster recovery in TLR4-/- and LPS-RS groups compared to vehicle group (*P<0.05 between groups, Kruskal Wallis test, n=6)

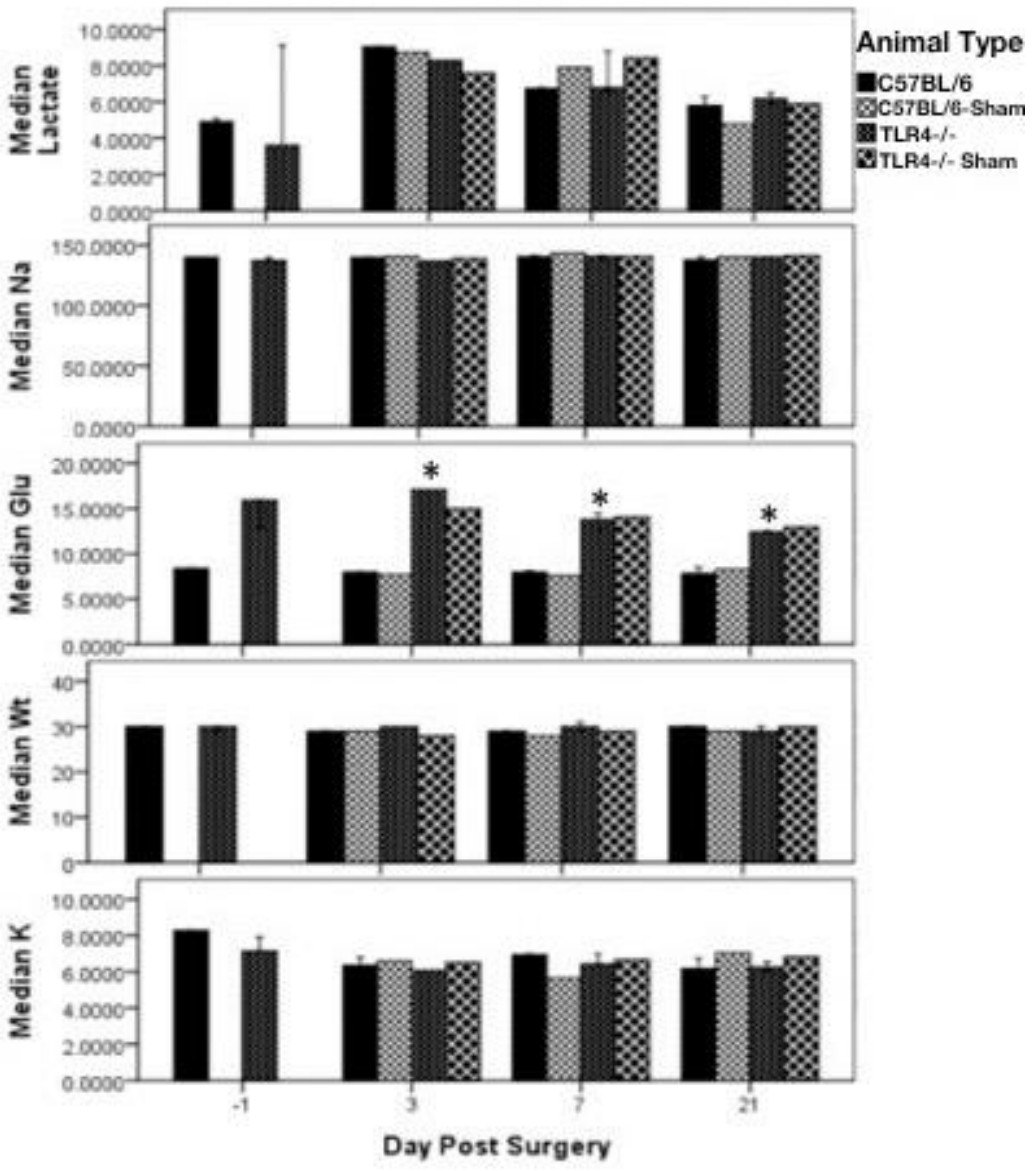


Figure 75 - Blood gas analysis of vehicle and TLR4-/- mice pre and post left hind-limb ischaemia and sham operation on day -1, 3, 7 and 21. Random blood glucose was significantly higher in TLR4-/- than CL57BL group (*P<0.05; Kruskal Wallis test, n=3).

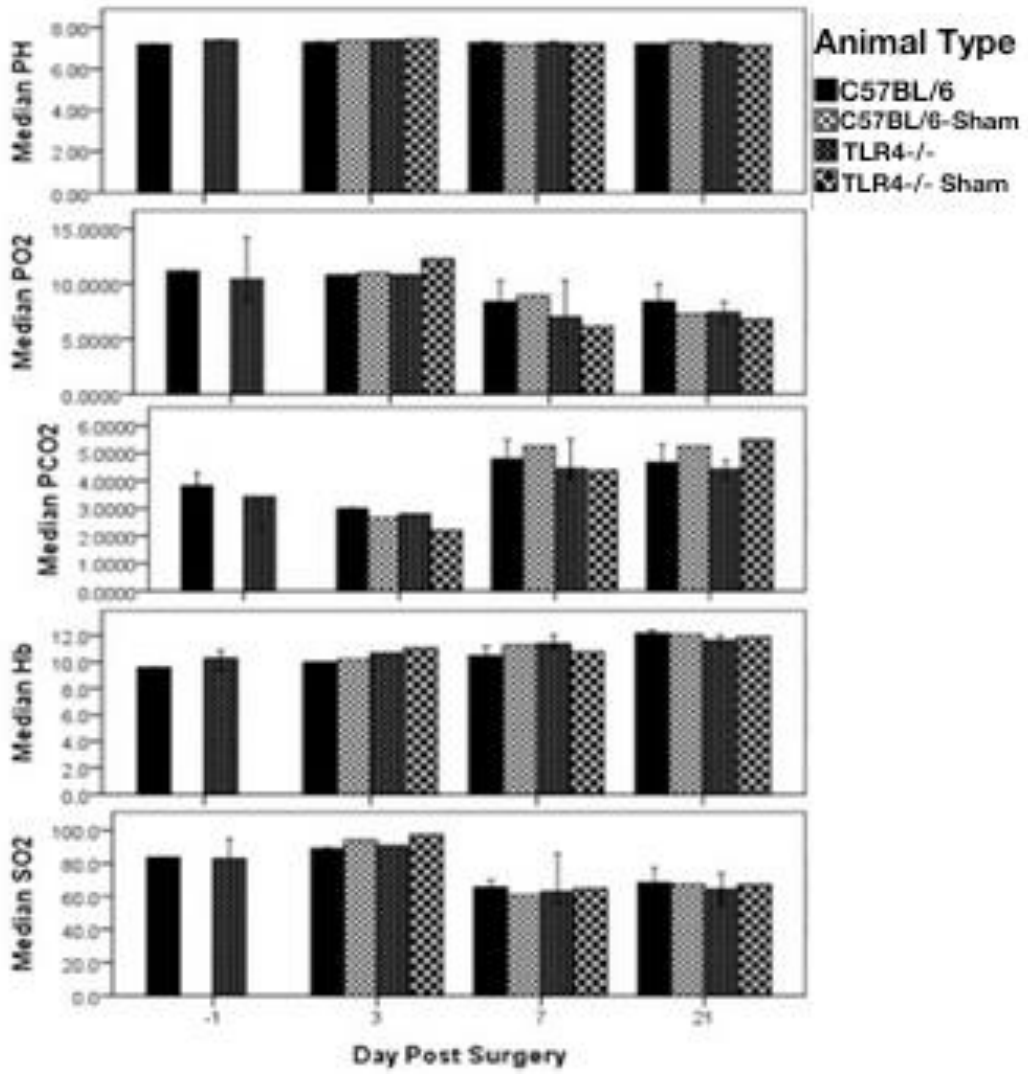


Figure 76 - Blood gas analysis of vehicle and TLR4-/- mice pre and post left hind-limb ischaemia and sham operation on day 3, 7 and 21 (No significant difference, Kruskal Wallis test, n=3)

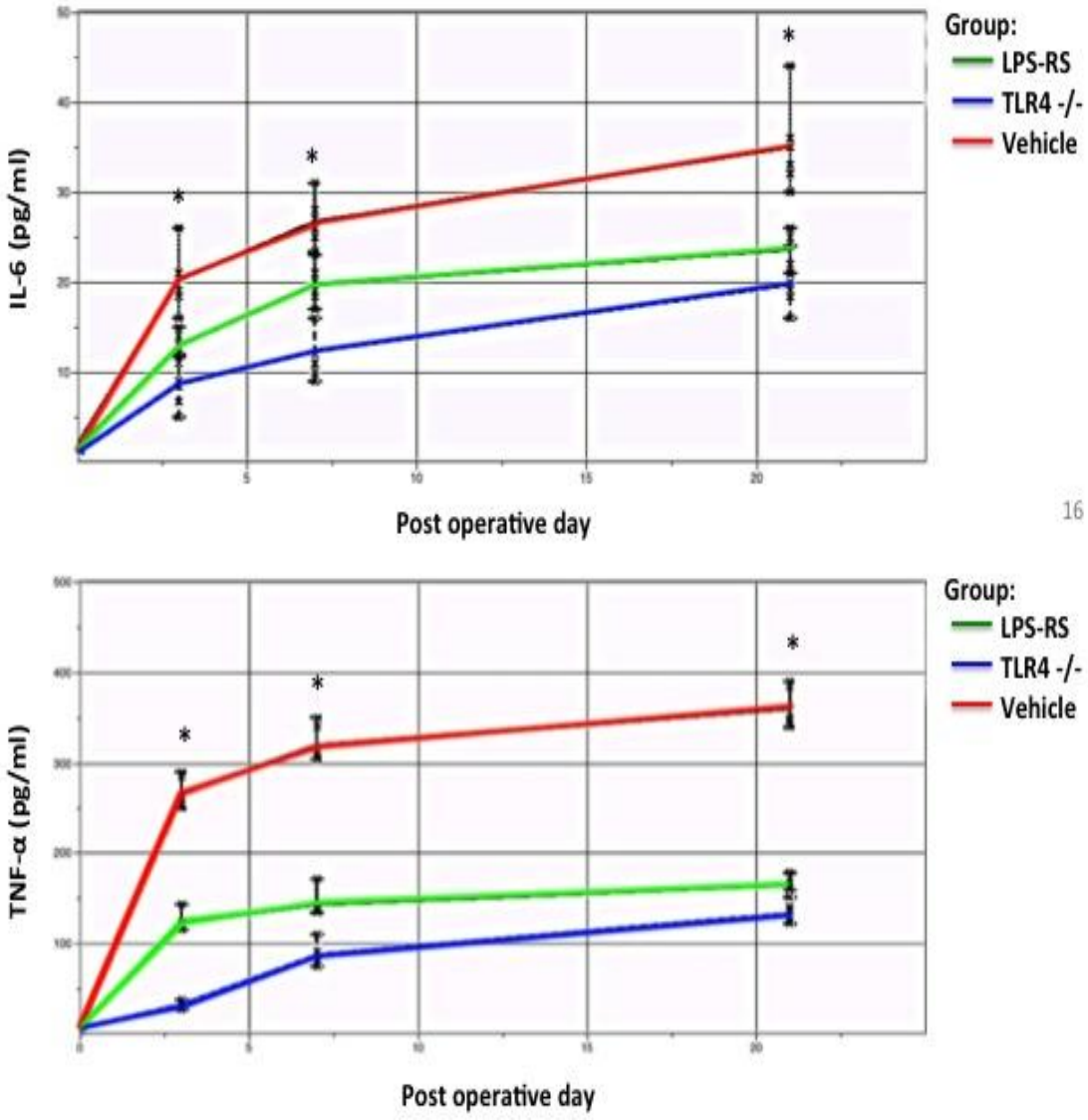


Figure 77 - IL-6 and TNFα levels in serum from vehicle, TLR4-/- and LPS-RS groups on day 7, 14 and 21 post operation. The IL-6 and TNFα levels were significantly different at each time point between the three groups (*P<0.05; Kruskal Wallis test, n=6)

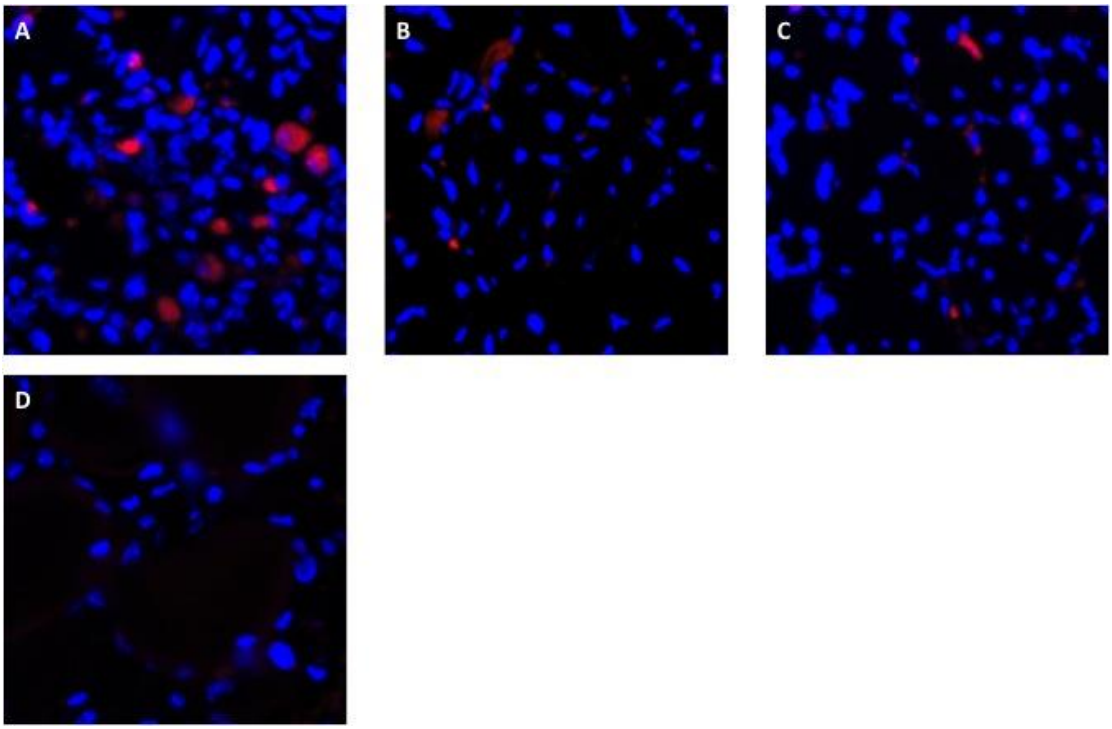


Figure 78 Fluorescent immunohistochemical staining for cleaved-caspase 3 in C57BL/6 mouse gastrocnemius muscle, post hind-limb ischaemia day 21 (Paraffin embedded, DAPI and AlexaFluor 647 stains, 3-micron thick section, x40 fluorescence); representative from A) Vehicle group; B) LPS-RS group; C) TLR4 -/- group; D) IgG (negative) control

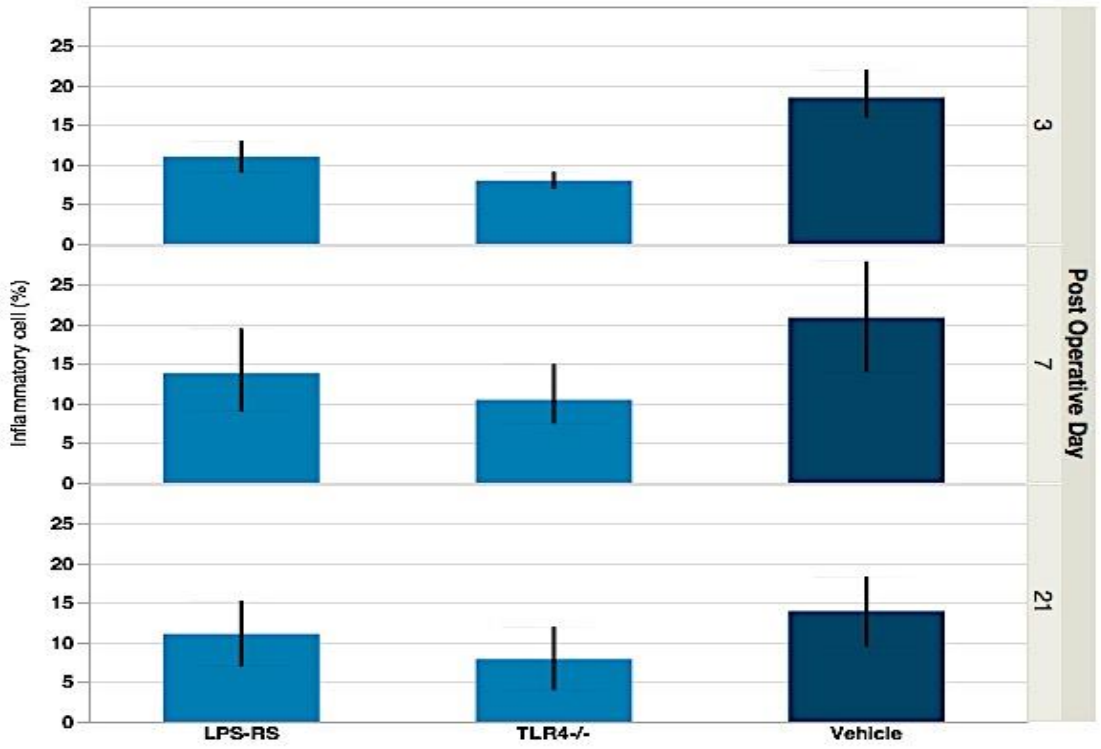


Figure 79 - Inflammatory cell quantification in H&E stained ischaemic gastrocnemius muscle showed less inflammatory cell infiltration in both TLR4 -/- and LPS-RS groups when compared to vehicle group at each time point (P<0.05; Kruskal Wallis test, n=6)

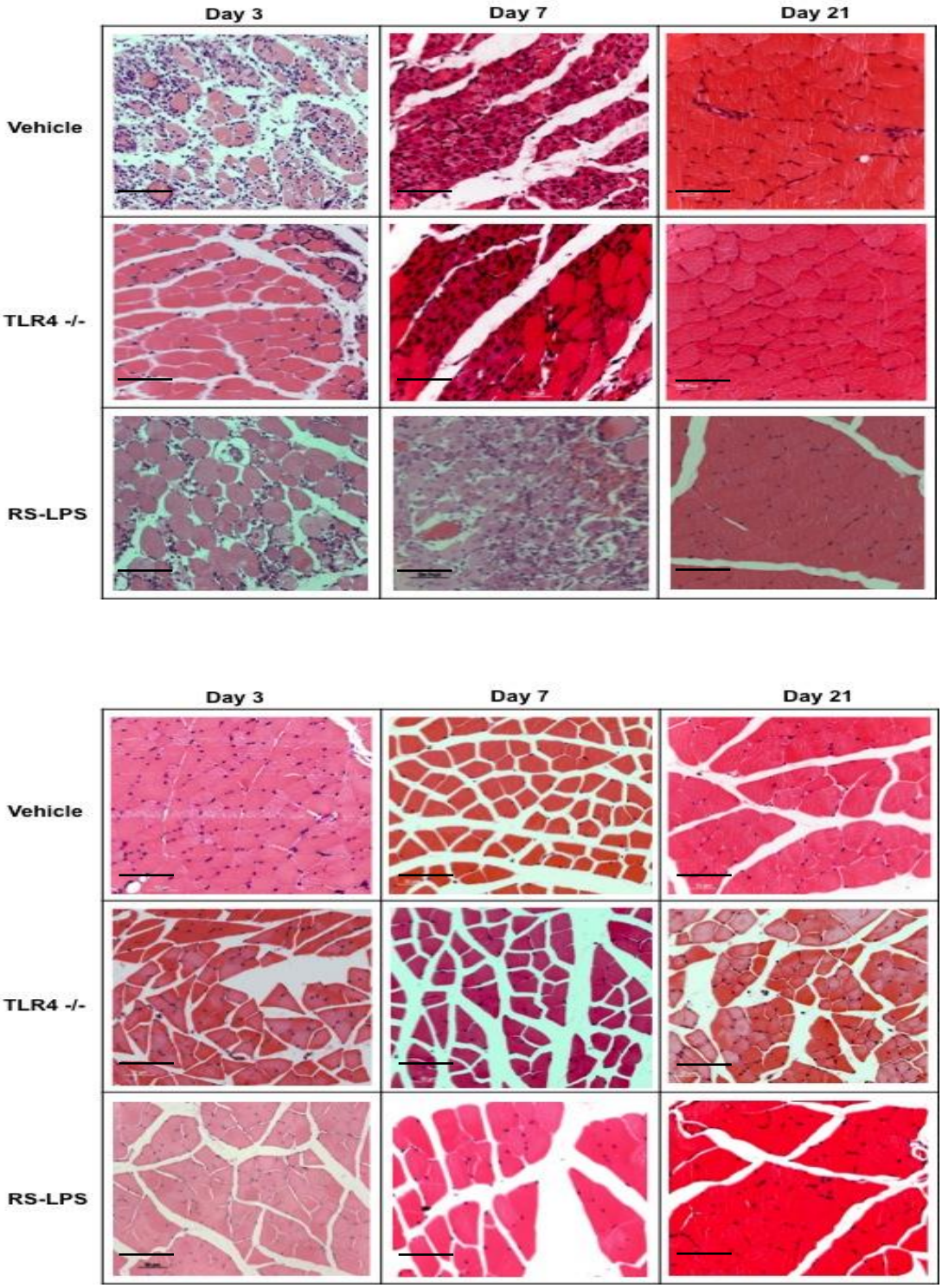


Figure 80 - Representative H&E staining of, ischaemic (top) and, non-ischaemic (bottom) gastrocnemius muscle post surgery day 3, 7 & 21 (scale bar = 50µm)

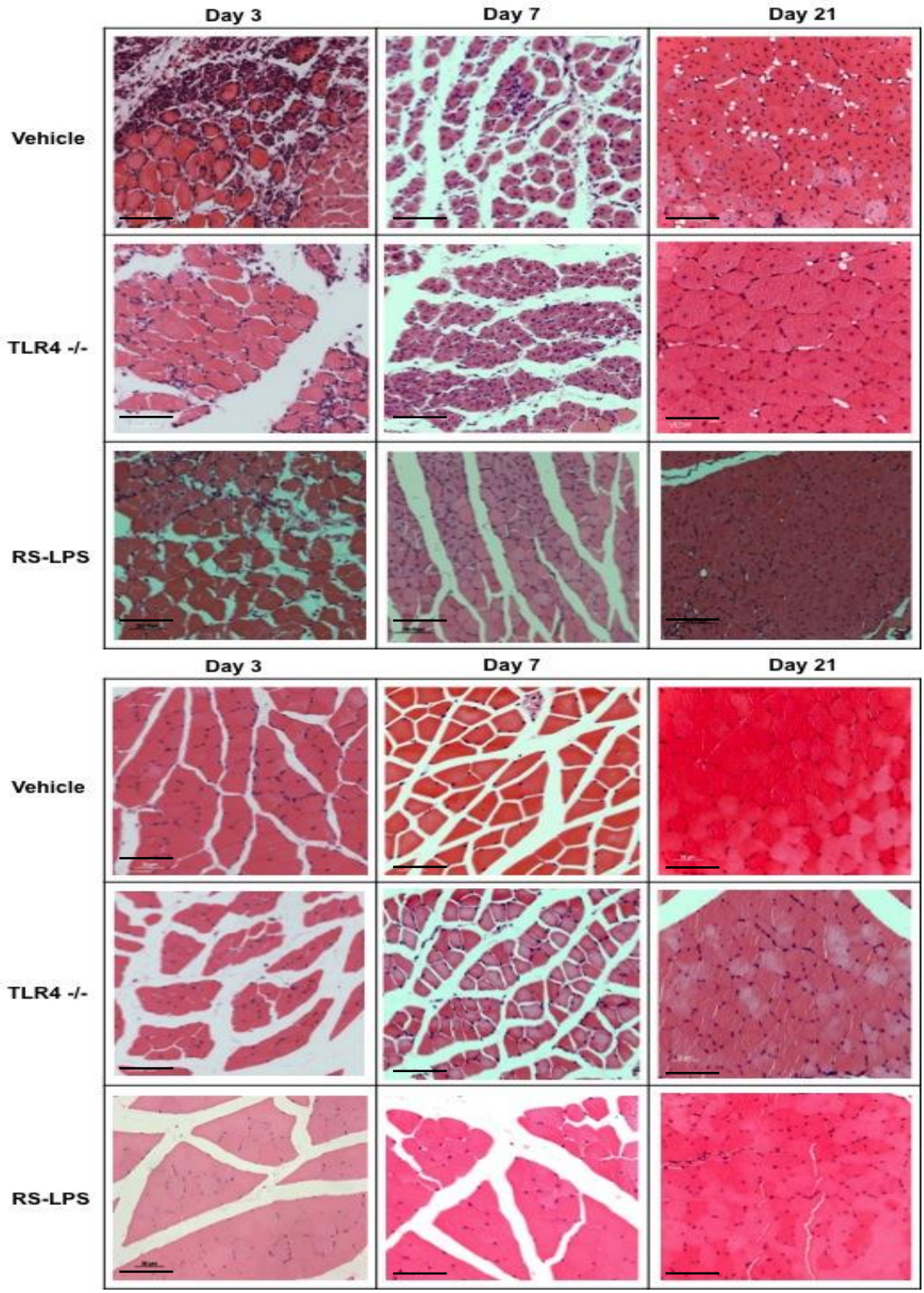


Figure 81 - Representative H&E staining of, ischaemic (top) and, non-ischaemic (bottom) tibialis anterior muscle post surgery day 3, 7 & 21 (scale bar = 50µm)

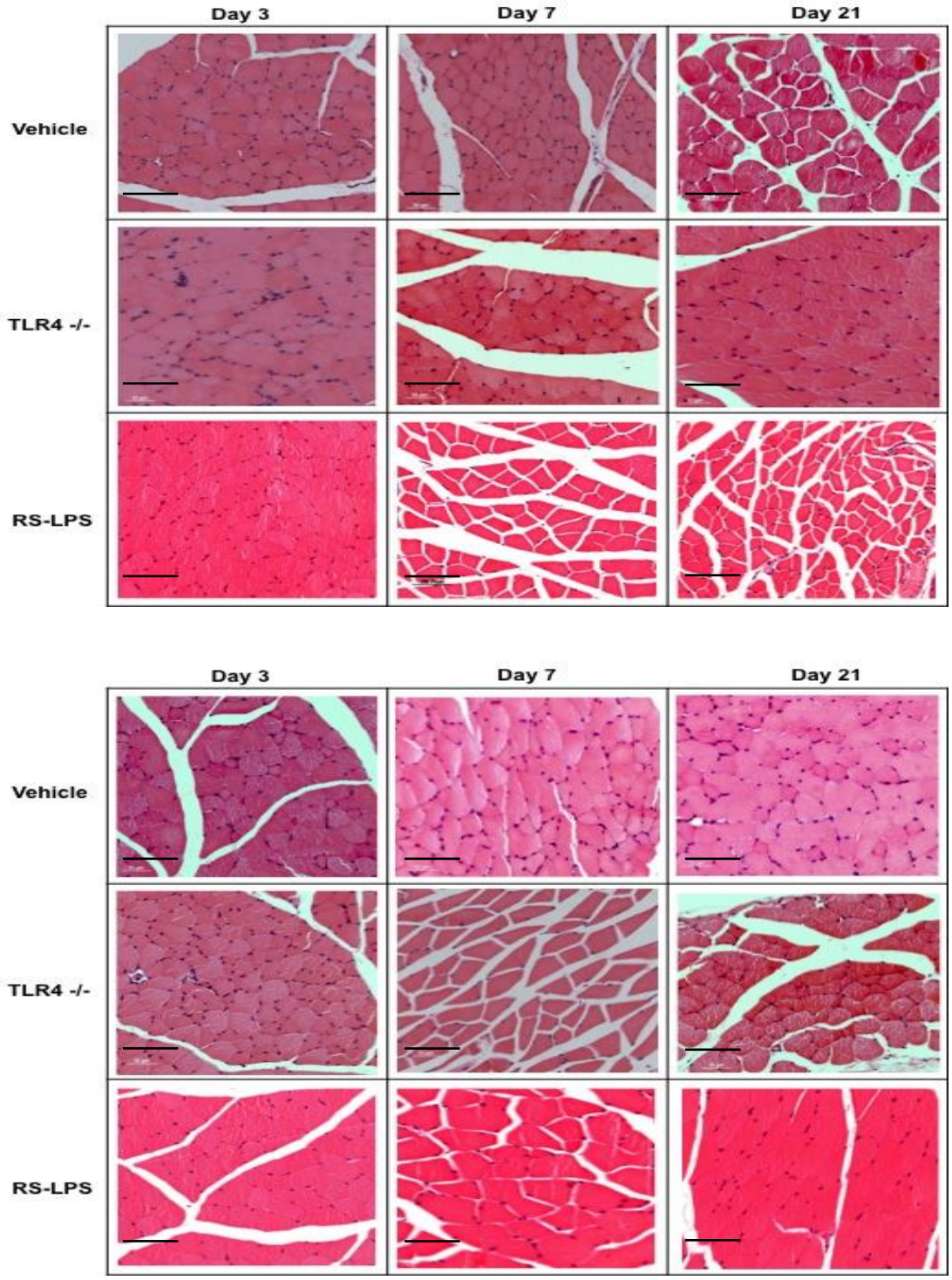


Figure 82 - Representative H&E staining of, sham operated (top) and, non-ischaemic (bottom) gastrocnemius muscle post sham surgery day 3, 7 & 21 (scale bar = 50µm)

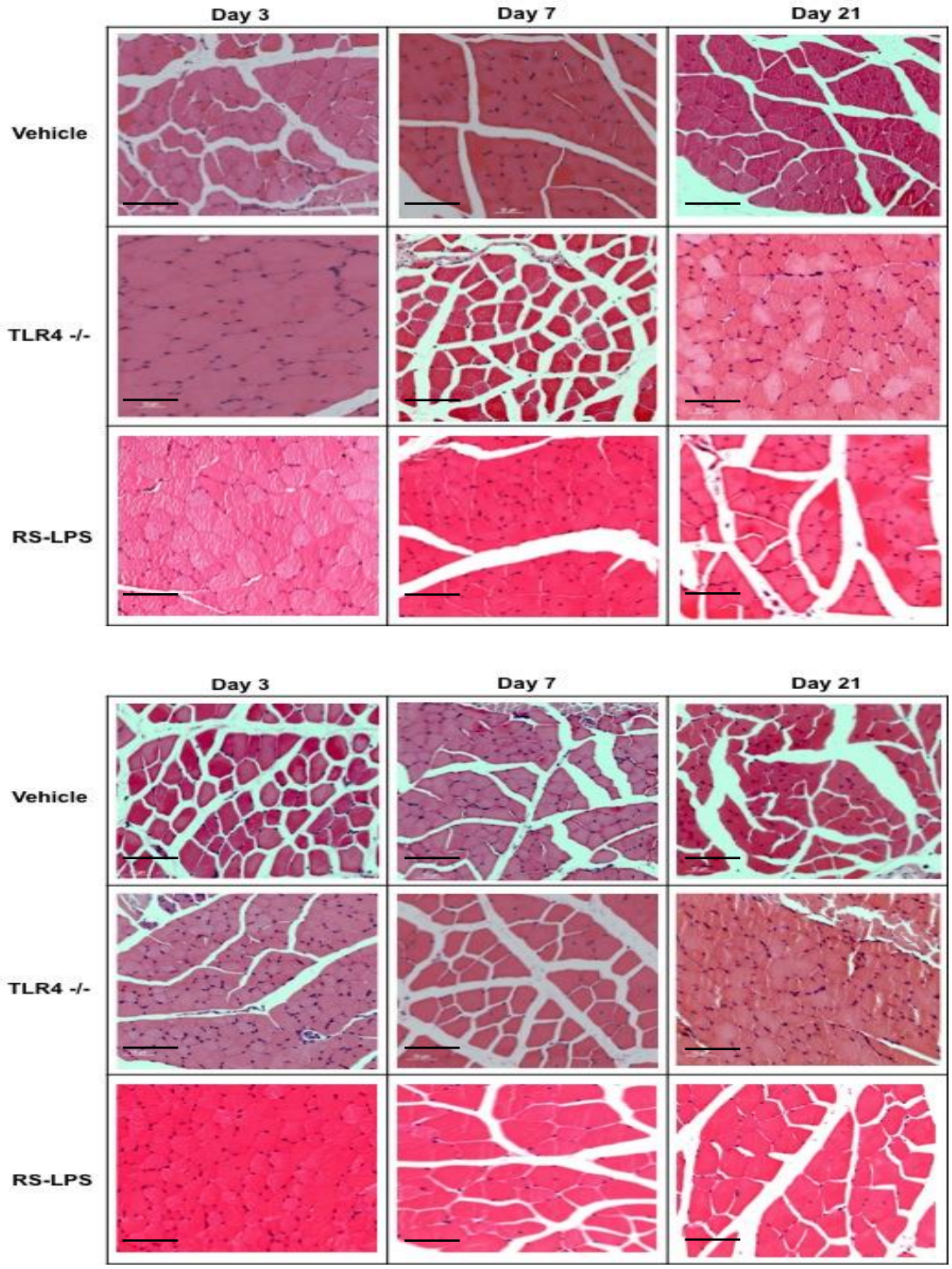


Figure 83 - Representative H&E staining of, sham operated (top) and, non-ischaemic (bottom) tibialis anterior muscle post sham surgery day 3, 7 & 21 (scale bar = 50µm)

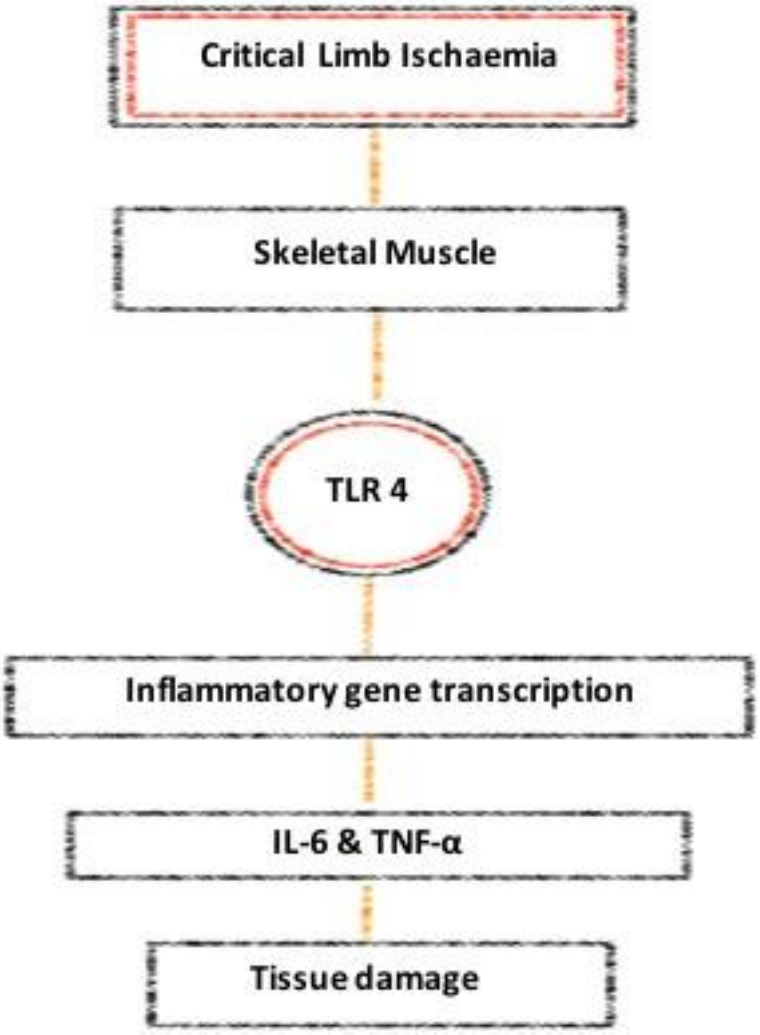


Figure 84 – Diagram suggesting the potential role of TLR4 in ischaemia-induced skeletal muscle damage

CHAPTER 7: GENERAL DISCUSSION

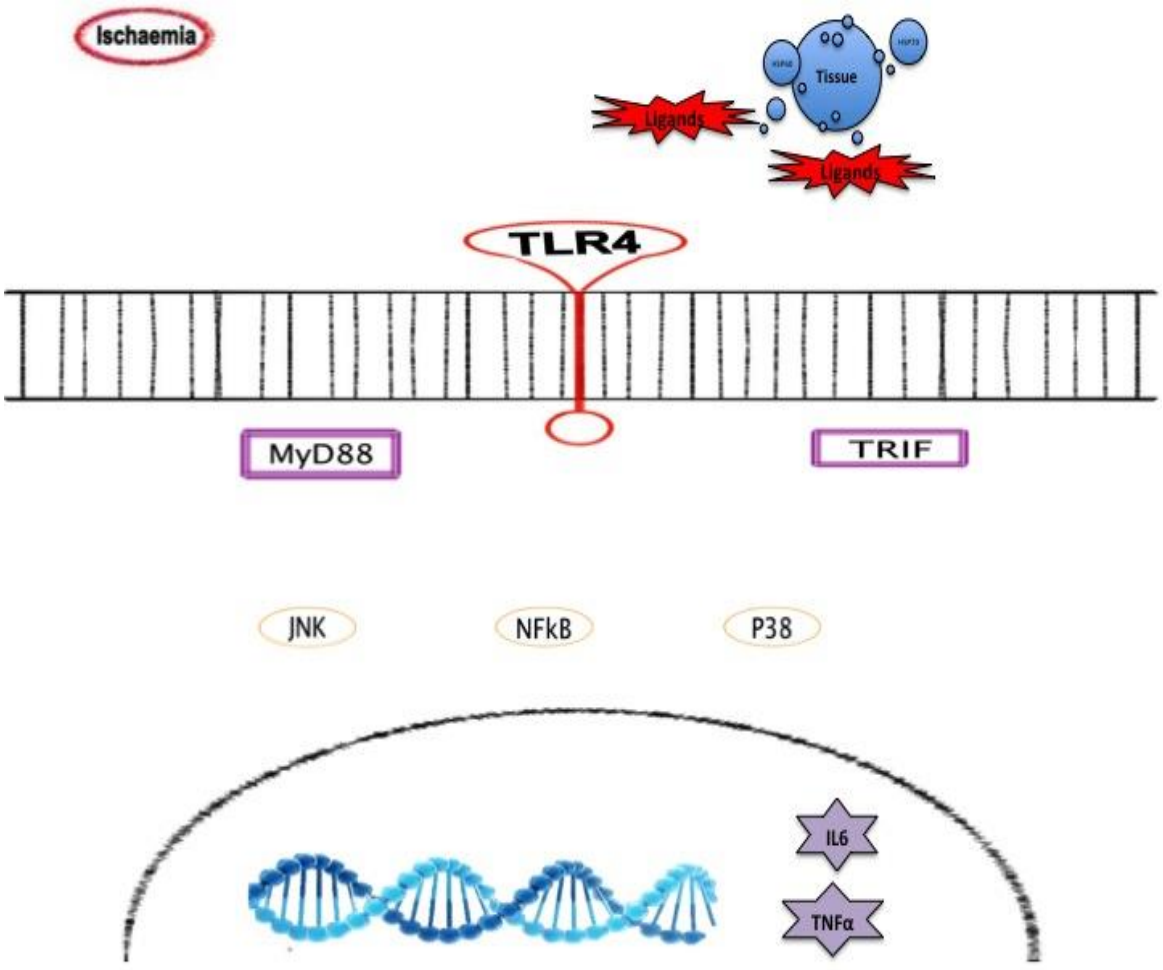
7. 1 Summary and discussion

7. 2 Limitations

7. 3 Future directions

7. 4 Potential clinical application

7. 5 Conclusions



7.1. Summary and discussion

The prevalence of CLI, impending limb loss that is caused by severe compromise of blood flow, is reported to be as high as 0.24% in the population aged between 40 and 69 years in Western society (248).The Vascular Society for Great Britain and Ireland estimated that 40 per 100,000 population experience CLI per year in the UK. An ageing population and improved survival from ischaemic heart disease and cerebrovascular events suggest that this incidence may be increasing. It has been reported that patients with CLI have mortality rates of 20% and 50% within one and five year respectively (249). More recent meta-analysis of mortality rates in patients with CLI estimated that probability of mortality was 3·7% at 30 days, 17·5% at 1 year, 35·1% at 3 years and 46·2% at 5 years (250). Another retrospective cohort study of patients who underwent a first major lower limb amputation showed frailty of this population, with almost 25% of patients dying within 30 days, and almost 50% at 1 year (251).

The main treatment options for patients with CLI remain limited to revascularisation, or amputation if the arterial tree is not re-constructible or tissue loss too severe. Advances in surgical technology has amplified the revascularization options by both open and endovascular approaches, however even revascularisation in critically ischaemic limbs is often not associated with significant improvement in functional status. These patients continue to have poor lower limb function and quality of life even though the distal blood flow is restored (252, 253). The poor functional outcomes have highlighted the need for consideration of other contributory factors involved in the pathophysiology of the CLI.

Primary amputation is considered for up to 20% of patients with CLI who have extensive tissue loss, or non-reconstructable disease. The European Consensus Document recommends primary amputation if it is deemed that the probability of saving a useful limb for the patient at 1 year is less than

25% (249). It has been shown that only 4% of patients who were in a dependent living situation preoperatively went on to live independently at 6 months, and only 21% who did not ambulate preoperatively were independently ambulatory 6 months after surgery (254).

Understanding the pathophysiology of the ischaemia-induced inflammatory damage in CLI may provide a potential missing link in improving functional outcomes along with successful revascularization. It is thought that the muscle dysfunction in CLI is potentially due to the ischaemia-induced skeletal muscle damage, in which inflammation plays a central role (18, 255).

The most common underlying aetiology of CLI is atherosclerosis, an inflammatory condition that increases the risk of cardiovascular events. The systemic levels of inflammatory cytokines have been shown to be increased in patients with CLI (256).

Vascular function is regulated by the endothelium and inflammatory-induced endothelial dysfunction is associated with increased risk of cardiovascular events. Inflammation is now a recognised risk factor for endothelial dysfunction: it increases oxidative stress, which is the common underlying factor of many of the known cardiovascular risk factors (257).

Hence, the systemic inflammatory response in patients with CLI can contribute to the higher prevalence of cardiovascular disease in this group of patients. Patients with PAD are six times more likely to die from cardiovascular event over 10 years than patients with no PAD (258).

The recent and intense interest around TLRs, has revealed that the innate immune system makes important contribution to the development of cardiovascular diseases, which are now recognized as chronic inflammatory conditions (259). TLRs are one of the key pattern recognition receptors in the innate immune system, and they have been shown to play an important role in ischaemia-induced inflammatory responses. There is mounting evidence that TLRs from both immune and non-immune cell origin are involved in the production of cytokines and inflammatory mediators in tissue ischaemia. This

has been widely investigated to explore the therapeutic potentials in pharmacological modulation of TLRs (42).

The indispensable role of TLR4-induced tissue damage in ischaemia has been shown in different organs such as brain, kidney, liver and myocardium (48, 238, 240, 242, 260). Earlier work by our group demonstrated the expression of TLRs in ischaemic muscle. TLR4 is also expressed in skeletal muscle where ischaemia-induced myopathy is associated with activation of the adaptive immune system (38).

In this work, expression of TLR4 was shown in both ischaemic and non-ischaemic human skeletal muscle, with increased expression in the ischaemic samples. The upregulation of TLR4 is in support of a notable role of TLR4 in the process of ischaemia-induced tissue damage. NFkB and JNK are key transcription factors in TLR4-mediated MyD88-dependent signalling pathway. TLR4-mediated NFkB and JNK pathways are shown to be involved in myocardium ischaemia (45, 261). Here it was shown that phosphorylated NFkB and JNK, activated forms of NFkB and JNK, were significantly up-regulated in the ischaemic samples. The high expression of these transcription proteins suggests that TLR4 is not only up-regulated but also activated. Further, the increase in cleaved caspase 3, a marker for apoptosis, suggests that significant tissue damage occurs even within clinically viable lower limb muscle in patients with CLI. This is in support of previous studies implicating TLR-induced apoptotic cell death in the pathophysiology of ischaemia-induced cell damage (38). Immunofluorescence staining of the muscle biopsies revealed the presence of TLR4 on neutrophils, endothelium and macrophages. These findings suggest wide distribution of TLR4 in the lower limb which may emphasis the role of these receptors in ischaemia-induced inflammatory response in patients with CLI.

To assess the significance of these findings in supporting the hypothesis, and to understand the downstream signalling pathway better, an *in vitro* model of ischaemic human myotube was utilised. This model, which was previously

developed by our group, showed that exposure of mature myotubes to nutrition depletion, hypoxia and hypercapnia simulated certain changes similar to skeletal muscle ischaemia *in vivo*. The overall effect of these changes was a significant time-dependent increase in the percentage of apoptotic cells and inflammatory cytokine release, which are features of skeletal muscle ischemia. This model is reproducible, easy to use and considers main characteristics of chronic ischaemia, including apoptosis, pO_2, pCO_2 and pH (139).

Using this model, simulated ischaemia in cultured human myotubes led to upregulation of TLR4 and activation of its downstream signalling pathway with increased cytokine release and apoptosis in the ischaemic human myotubes. Further, inhibition of TLR4 prior to ischaemia was associated with inhibition of the signalling pathway and reduced ischaemia-induced apoptosis. Moreover, amplified cytokine release and increased expression of HSP60 and 70 was demonstrated in ischaemic skeletal muscle *in vitro*. Inhibition of the MyD88-dependent signalling pathway attenuated inflammatory cytokine production in the presence of ischaemia-induced TLR4 activation. This data is in keeping with previous findings of accelerated muscle regeneration in hind-limb ischaemia in MyD88 knock out mice as described previously (262) and supports the involvement of MyD88 in the inflammatory response to ischaemia, which may be a critical step in the development of skeletal muscle damage. Furthermore, TLR4 antagonism was associated with reduced inflammatory cytokine release and down-regulation of HSP60 and 70 expressions. This suggests a potential pathway where TLR4 and its endogenous ligands contribute to a positive feedback loop to maintain a pro-inflammatory environment during ischaemia.

The murine model of hind-limb ischaemia used in this work involved the excision of the common and superficial femoral arteries with ligation of the side branches in male C57BL/6 mice of 12 weeks of age, which resulted in reproducible ischaemia-induced skeletal muscle changes that closely mimics

those that occur in patients with CLI. This provides a useful pre-clinical model for further work including the assessment of novel muscle-protective adjunctive therapies for the treatment of CLI. In this *in vivo* model, it was shown that TLR4 is up-regulated in ischaemic skeletal muscle. TLR4-/- mice and mice given LPS-RS, a TLR4 antagonist, exhibited reduced systemic IL6 and TNFα levels following hind-limb ischaemia, implicating the role of TLR4 in ischaemia-induced systemic inflammatory cytokine production. Both endogenous and exogenous inhibition of TLR4 were associated with reduced inflammatory cell infiltration and diminished apoptosis in the ischaemic limb as compared to the control group. These findings suggest that TLR4 plays a key role in ischaemia-induced skeletal muscle damage. In previous studies, it has been argued that the TLR-induced inflammatory response plays a critical role in angiogenesis in tissue ischaemia, however the lack of TLR4 has been shown to have no negative effect on this process in this model (262, 263).

Patients with PAD have two to three fold increase risk of five to ten year cardiovascular related mortality. PAD appears to increase the risk of myocardial infarction and perhaps stroke (264). This increased risk might be due to systemic inflammatory response induced by PAD, with lower limbs as the main source.

7.2. Limitations

Muscle biopsies in the control group (No PAD) were obtained from patients undergoing CABG. Since coronary artery disease shares the roots of its pathophysiology with PAD, the patients were selected only if they had no history of lower limb arterial claudication with normal ABPI measurements. It is still possible for the patients in the control group to have subclinical PAD affecting the results of the human tissue experiments. Further, the number of human tissue biopsies was limited by time and resources.

In human tissue biopsies, the two main adherent cell types obtained from

enzymatic digestion are myogenic cells and muscle-derived fibroblasts. In our experiments, we used muscle homogenates to quantify the proteins. This approach does not allow identification of the cell types associated with the relevant proteins. Therefore, the results indicated protein expression in tissue rather than specific cell types involved. The presence of potential infection in the biopsied skeletal muscle is another limitation of our experiments. Infecting microorganisms are potent TLR4 activators (265) and therefore the presence of any infection could have interfered with the results. To minimize the potential infection-related bias, all the ischaemic biopsies were taken from sites away from ulcers and grossly necrotic tissue.

Although the activation of NFkB and JNK is in favour of TLR4 activation, many other immune receptors share these proteins in their downstream signalling pathways (266) and therefore more detailed experiments are required to confirm the implications of these findings and indeed this needs to be considered in the development of potential therapeutic strategies.

In the *in vitro* experiments, despite regular observations of the cell lines and application of antibiotics, the possibility of contamination of the cultured muscle cells with other cell lines or infective microorganisms, could have affected the results.

In the *in vivo* experiments, the limited number of RNA experiments made it difficult to confirm the statistical significance in the RNA work, however the results showed clear up-regulation of the TLR4 in the control group with induced hind-limb ischaemia. In addition, the overall sample size was kept to minimum to reduce the number of required animals.

The laser Doppler imaging allows quantitative analysis of functional blood flow, while it lacks anatomical information. The new advances in small animal imaging such as micro-CT provides the opportunity to achieve more anatomical information while the animal can be imaged multiple times non-invasively over the period of the study (267).

The overall limitation of this study is the need for more in depth analysis of the

signalling pathways and mechanisms involved, as the crosstalk between different signalling pathways can affect the final response. Also, additional work is needed to investigate the optimal time and delivery method of the TLR4 inhibition in more detail.

7.3. Future directions

Although our model of hind-limb ischaemia closely mimics the skeletal muscle changes which occur in patients with CLI, further work is required to optimise the model to exhibit more chronically induced changes. This will also allow the assessment of the pathophysiological changes in more detail with longer follow up. This can also be developed in other species for further validation of the results before the translation of the outcomes into human trials.

Further studies are required to explore different routes and timing of drug delivery and the dose response effect of TLR4 antagonists. Considering the positive effects of TLR4 activation in stimulating the necessary inflammatory response, the aim would be to only control the unnecessary immune response or to prevent the ongoing inflammatory damage without affecting the protective aspects of the process. Although TLR antagonists have clearly been shown to be protective by directly inhibiting inflammatory gene transcription and reducing secretion of inflammatory cytokines in several conditions, the preconditioning with low dose TLR agonists may also be an effective strategy to protect tissue against subsequent detrimental insults such as reperfusion injury. This highlights the importance of administering specific TLR agonists and antagonists during the relevant phase of the pathological process in order to achieve an optimal balance of TLR activation and inhibition (42).

TLR4 can interplay with other inflammatory signalling pathways to balance the final response to ischaemia. The inflammation is required for both muscle

regeneration and angiogenesis, however, a regulated balance is required to prevent unnecessary inflammation-induced tissue damage, while preserving the positive effects. It has been reported that unopposed TLR4 activation in skeletal muscle ischaemia slows regeneration and also angiogenesis; while TLR2, which is simultaneously up-regulated by TLR4, plays a protective role to modify the TLR4 effect (183). Further, the potential of TLR4 and TLR1 heterodimerisation needs to be explored, where TLR1 activation may affect the final response to the tissue ischaemia and confers a degree of specificity in the response.

TLR4 response to simulated ischaemia is mainly regulated via MyD88 signalling pathway, which results in activation of transcription proteins (NFkB, JNK and P38). The inhibition of these regulatory proteins is associated with less inflammatory response. Hence, pharmacological interruption of the signalling pathway further downstream can be of clinical significance. However, this needs careful evaluation as these regulatory proteins are shared between many other signalling pathways, and therefore their inhibition can be associated with some adverse effects.

Moreover, the other routes of adaptive immune system such as T-cell mediated response are further potential areas of future research to explore the possible interactions with TLR4 signalling pathway.

7.4. Potential clinical application

Revascularisation, as per current practice, offers the best option for limb salvage. The poor functional outcomes, even after successful revascularization, highlight the clinical need to developing adjuvant therapies alongside surgical intervention.

Therapeutic angiogenesis is another novel concept to treat patients with CLI. This involves the use of angiogenic growth factors or stem cells, in order to stimulate blood vessel growth to augment perfusion. Clinical trials on the

application of angiogenic growth factors have showed potential to promote tissue angiogenesis in limb musculature in patients with CLI. However, this requires multiple treatments over months. Also, therapeutic outcomes are still limited and further improvements are required for extensive clinical applications (268). There are a number of pre-clinical studies showing beneficial effects of vascular stem cell therapy in ischaemic disease, however translation of these findings to the clinic is challenging. Cell therapy products are manufactured under strict, centralized procedures resulting in extra costs and more complex regulatory pathway than synthetic drugs (269).

Our study suggests that ischaemia induces an inflammatory response in skeletal muscle, which then contributes to the underlying skeletal muscle dysfunction. Hence, pharmaceutical inhibition of TLR4 might be of clinical significance in patients with CLI. The TLR4 inhibition can also be considered for the management of earlier stages of PAD when patients present with claudication. The reversal of the ischaemia-induced myopathy might be more feasible in the earlier stages of the disease.

The inhibition of TLR4 can also be considered in adjunction with other novel strategies. Reducing the ischaemia-induced inflammatory response may improve the environment/ niche to enable growth factors or stem cells to function better.

7.5. Conclusions

This study investigated the role of TLR4-mediated inflammatory response in critically ischaemic skeletal muscle. Human myotubes derived from patients with and without PAD were successfully cultured *in vitro* and a mouse model of hind-limb ischaemia suitable for evaluating ischaemic muscle damage was developed. TLR4 was found to be up-regulated and activated in ischaemic skeletal muscle in patients with CLI, and TLR4 signalling contributed to ischaemia-induced inflammation and cell/tissue damage *in vitro* and *in vivo*.

Modulating TLR4 signalling *in vivo* was associated with attenuation of skeletal muscle damage. This work highlights the potential therapeutic role of TLR4 inhibition in patients with CLI.

It is conceivable that pharmacological targeting of TLR4 in patients with CLI along with improving the haemodynamics may improve both functional and clinical outcomes.

APPENDICES

1. **Visiting scholarship at Yale University**
    - Acute model of hind-limb ischaemia
    - Chronic model of hind-limb ischaemia
2. **Search strategy**
3. **List of awards, publication and presentations**
4. **Publications and presentations**
5. **Patient information sheet, consent form and data collection form**
6. **Ethics approval and personal licence**
7. **Levels of evidence**

1. Visiting scholarship at Yale University

Based on the work for this thesis, I was granted a Bogue Fellowship for a 4-week-visit to Prof Alan Dardik's lab at Yale University, New Haven, USA. This was complimented by a one day visit to Dr Louis Messina's lab at University of Massachusetts Medical School. The purpose of this visit was to observe the locally developed models of hind-limb ischaemia.

The Bogue Fellowship is provided by a bequest from the late James Yule Bogue, a former Research Fellow in the UCL Department of Physiology and one time Deputy Chairman of the Pharmaceuticals Division of ICI. The fellowship supports visits to carry out research in laboratories in the USA and Canada in order 'to enrich the research experience and help develop the scientific career of the Fellow'.

Professor Dardik is a surgeon-scientist who seeks to use the basic science laboratory to perform cutting edge research to ultimately benefit patients with vascular disease.

- Acute model of hind-limb ischaemia

Alan Dardik developed a mouse model of hind-limb ischaemia to study therapeutic angiogenesis (246). In this model, unilateral high femoral artery ligation and superficial femoral artery excision were performed on 6-8 weeks old male C57BL/6 mice [Figure 85]. Intraperitoneal anaesthesia was administered using ketamine (100 mg/ kg) and xylazine (5 mg/kg). Mice were positioned in dorsal recumbency with their hind limbs externally rotated. A skin incision was made over the femoral artery beginning at the inguinal ligament and continued caudally to the popliteal bifurcation. The femoral artery was isolated above the level of the profunda and epigastric arterial branches, doubly ligated using 7-0 Prolene suture, and transected. The superficial femoral artery caudal to the major branch points was dissected, ligated, and excised in its entirety [Figure 86, Figure 87].

This model closely resembles diseases in humans. In comparison to other models, the common femoral artery and its proximal deeper branches in this model are occluded but still present. Extensive excision of the arterial tree can adversely affect the angiogenesis. This model resulted in signs ranging from no necrosis to toe necrosis, in comparison to the models with more severe ischaemic damage with profound necrosis.

By visiting Dardik's lab, further experience was gained in application of Duplex technology to assess haemodynamics post surgery. However, this was not translated into our project due to logistical problems [Figure 88].

Louis Messina investigated the effect of gradual or acute arterial occlusion on skeletal muscle blood flow, arteriogenesis, and inflammation in rat hind-limb ischaemia model (270), It is argued that sudden interruption in the blood flow will result in increased shear forces proximal to the ligation, and this will directly affects collateral formation. Also, it is argued that acute ischaemia is associated with more tissue necrosis and aggravated inflammatory response which again contributes to the angiogenesis. In the Messina's rat model of acute ischaemia, hind-limb ischaemia was created by ligation of the left common iliac artery and left femoral artery and all their branches [Figure 89].

- Chronic model of hind-limb ischaemia

  In the rat model of chronic ischaemia, a similar operation to the acute model was performed in Messina's lab, but instead of ligating the left common iliac artery at the aortic bifurcation and the left femoral artery above the saphenopopliteal bifurcation, a 0.75-mm internal diameter ameroid constrictor was placed around the artery at each location. Ameroid constrictors have an outer metal sleeve encasing an inner layer of a hygroscopic material, usually casein, and when placed around an artery they induce gradual vessel occlusion over 2-3 days as they absorb moisture from the surrounding tissues. These custom ameroid constrictors were sterilized with ethylene gas prior to implantation. No arteriotomies were performed [Figure 90].

The model of gradual ischaemia showed signs of chronic ischaemia but failed to reproduce critical limb ischaemia. In this group, rats showed muscle atrophy, but no evidence of necrosis or limping, when compared to Messina's acute model.

The chronic model of ischaemia was associated with reduction in the blood flow, with less inflammatory changes in the skeletal muscle. The rats that were treated by gradual arterial occlusion showed preserved muscle mass and architecture without muscle necrosis or late fibrosis.

2. Search strategy

Developing a well-built research question is fundamental to retrieving the relevant research literature. It is advocated that a well-built question should include four parts, referred to as PICO that identify the patient problem or population (P), intervention (I), comparison (C) and outcome (O) (271). The PICO framework is applied to the dissertation question as described below:

PICO Framework	
Patients / In vitro / In Vivo	CLI / Skeletal muscle ischaemia / TLR Expression
Interventions	Induced Ischaemia / Systemic deficiency OR Exogenous inhibition of specific TLRs
Comparison	No modification to TLRs
Outcome	Tissue damage in lower limb ischaemia

The primary search was performed by considering the first two elements of PICO (Patients and Interventions). As per result of the primary search, further components of PICO were added to refine the search.

Literature was reviewed by searching the following databases: MEDLINE, EMBASE, Cochrane Library and Google search. OvidSP and PubMed were used as database gateways. All the relevant research literature can be retrieved from electronic databases with close to perfect sensitivity or specificity, or with high precision, by using comprehensive search strategies. To construct a comprehensive set of search terms, a combination of MeSH terms and free texts referring to subject headings and subheadings, is advocated (272). Therefore, multiple Mesh terms and free texts referring to the subject headings and subheadings were used to identify the relevant evidences as below:

The search strategy			
Key terms	**MeSH**	**Limitations**	**Databases**
TLR **Skeletal muscle** **Myopathy** **In vivo** **Mouse module** **Chronic limb** **Ischaemia** **Critical limb** **ischaemia** **Induced ischaemia** **Tissue damage** **DAMP** **Endogenous** **ligands** **TLR antagonism**	**Drug Therapy** **Autoimmune** **Disease** **In Vitro** **Animal** **Experimentation** **Drug Antagonism** **Peripheral arterial** **disease**	**2001 - 2017** **English** **language** **Grey literature**	**MEDLINE** **EMBASE** **Cochrane** **Library** **Google**

The above keywords will be used in different combinations to increase the both sensitivity and specificity of the search. In order to include more evidence, articles published within the last 10 years are considered. Also, only the articles written in English are included to refine the search. Back chaining retrieval through the references of the electronically obtained evidences was applied to include more articles.

The grey literature was not searched as a major limitation of the grey literature is that many of the studies reported are initiated in response to a political problem but with inadequate funding and unavoidable limitations that seriously undermine the credibility and value of the final product (Committee on Environmental Epidemiology, National Research Council. 1997).

3. List of Awards, Publications and Presentations

Awards

- PVD Travel Award ($500) - Awarded by American Heart Association (AHA); Arteriosclerosis, Thrombosis and Vascular Biology (ATVB) conference in Toronto; May 2014

- Travel bursary (£100) - Awarded by Society of Academic & Research Surgery; SARS annual meeting in Cambridge; January 2014

- Travel Scholarship ($1000) - Awarded by Society for Vascular Surgery (SVS) Research and Education Committee; Vascular Research Initiatives Conference and the Arteriosclerosis, Thrombosis and Vascular Biology meeting in Orlando; April 2013

- Bogue Research Fellowship (£2,500) - Awarded by University College London for 4 weeks visit at Dardik's lab at Yale University; December 2012

Publications

- Navi A, Patel H, Shaw S, Baker D, Tsui J. Therapeutic Role of Toll-Like Receptor Modification in Cardiovascular Dysfunction. *Vascular Pharmacology.* 2012: 58(3): 231-9. [23070056]

Published abstract

- Navi A, Yu R, Abraham D, Shi-Wen X, Baker, Tsui J, Mouse Model of Ischaemia-induced Skeletal Muscle Damage. *European Journal of Vascular and Endovascular Surgery*, 2014; In Press

- Navi A, Abraham D, Shi-Wen X, Hamilton G, Baker D, Tsui J. Critical Role of Toll-like Receptor 4 (TLR4) in Ischaemia-induced Skeletal Muscle Damage*. ATVB.* 2014; In Press

- Navi A, Abraham D, Shi-Wen X, Shaw S, Baker D, Tsui J. Role of Toll-like receptor 4 (TLR4) inhibition in ischaemia-induced systemic inflammatory response and skeletal muscle damage. *British Journal of Surgery*, 2014; 100: 31

- Navi A, Abraham D, Shi-Wen X, Shaw S, Baker D, Tsui J. Toll-like receptor 4 (TLR4) inhibition reduces ischaemia-induced skeletal muscle damage. *British Journal of Surgery*, 2013

- Navi A, Yu R, Shi-wen X, Shaw S, Hamilton G, Baker D, Tsui J. Detrimental role of Heat Shock Protein 60&70 and Toll-like Receptor 4 in skeletal muscle ischaemia *in vitro*. *Journal of Vascular Surgery*. 2013; 57(5): 77

- Navi A, Shi-wen X, Shaw S, Hamilton G, Tsui J. Emerging Role of Toll-like Receptor 4 and Heat Shock Protein 60&70 in Ischaemia-Induced Skeletal Muscle Damage In Vitro. Arterioscler Thromb Vasc Biol. 2013; 33: A197

- Navi A, Yu R, Shi-wen X, Shaw S, Baker D, Tsui J. Detrimental role of MyD88-dependent signaling and Toll-like receptor 4 in ischaemia induced skeletal muscle damage in vitro. *European Journal of Vascular and Endovascular Surgery*, 2013; In Press

- Navi A, Patel H, Abraham D, Shi-Wen X, Baker D, Tsui J. Functional consequences of ischaemia-induced Toll-like Receptor 4 (TLR4) activation in skeletal muscle. *British Journal of Surgery*, 2013; 100: 15

- Navi A, Abraham D, Shi-Wen X, Shaw S, Baker D, Tsui J. Functional up-regulation of toll-like receptor 4 and 1 in ischaemic skeletal muscle. *Cardiovascular Pathology*, 2013; 22(3): 31

- Patel H, Navi A, Shi-Wen X, Abraham D, Baker D,, Shaw S, Tsui J. Toll-like receptor (TLR) 2 and 6 ligands and heat shock proteins 60 and 70 may play a role in the pathogenesis of skeletal muscle damage in critical limb ischaemia. *Cardiovascular Pathology*, 2013; 22(3): 3

Oral Presentations

- Critical Role of Toll-like Receptor 4 (TLR4) in Ischaemia-induced Skeletal Muscle Damage. Society for Vascular Surgery (SVS) Vascular Research Initiatives Conference, Toronto, Canada, April 2014

- Role of Toll-like receptor 4 (TLR4) inhibition in ischaemia-induced systemic inflammatory response and skeletal muscle damage. Society of Academic and Research Surgery (SARS), Cambridge, January 2014

- Detrimental role of MyD88-dependent signaling and Toll-like receptor 4 in ischaemia induced skeletal muscle damage in vitro. European Society for

Vascular Surgery, Frankfurt, Germany, May 2013

- Emerging Role of Toll-like Receptor 4 and Heat Shock Protein 60&70 in Ischaemia-Induced Skeletal Muscle Damage In Vitro. Society for Vascular Surgery (SVS) Vascular Research Initiatives Conference, Florida, USA, April 2013
- Functional consequences of ischaemia-induced Toll-like Receptor 4 (TLR4) activation in skeletal muscle. The Vascular Society AGM, Manchester, November 2012

Poster Presentations

- Mouse Model of Ischaemia-induced Skeletal Muscle Damage. European Society for Vascular Surgery, London, May 2014

- Critical Role of Toll-like Receptor 4 (TLR4) in Ischaemia-induced Skeletal Muscle Damage. Arteriosclerosis, Thrombosis, and Vascular Biology Scientific Sessions Toronto, Canada, April 2014

- Toll-like receptor 4 (TLR4) inhibition reduces ischaemia-induced skeletal muscle damage. *Vascular Society (AGM),* Manchester, November 2013

- Detrimental role of Heat Shock Protein 60&70 and Toll-like Receptor 4 in skeletal muscle ischaemia in vitro. Society for Vascular Surgery (SVS) annual meeting, San Francisco, USA, June 2013

- Emerging role of Toll-like Receptor 4 and Heat Shock Protein 60&70 in ischaemia-induced skeletal muscle damage *in vitro.* Arteriosclerosis, Thrombosis, and Vascular Biology Scientific Sessions, Florida, May 2013

- Functional Upregulation of Toll-like Receptor 4 and 1 in Ischaemic Skeletal

Muscle. International Society of Applied Cardiovascular Biology (ISACB), London, September 2012.

- The Toll-like Receptor (TLR) 2 and 6 ligands Heat shock proteins (HSP) 60 and 70 may play a role in the pathogenesis of skeletal muscle damage in critical limb ischaemia (CLI). International Society of Applied Cardiovascular Biology (ISACB), London, September 2012.

4. Publications and presentations

Functional upregulation of Toll-like Receptor 4 and 1 in Ischaemic Skeletal Muscle

Ali Navi, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Sidney G. Shaw**, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **Janice Tsui**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK;

Purpose: It has been suggested that the inflammation induced by ischaemia in peripheral arterial disease (PAD) has a key role in damaging lower limb skeletal muscle. TLR4 has been implicated in ischaemia and has been shown to form heterodimers with TLR 1. We aimed to study expression, activation and distribution of TLR4&1 in human critical limb ischaemia (CLI). **Methods:** Human skeletal muscle biopsies were taken from the medial head of gastrocnemius from patients undergoing major lower limb amputation due to CLI (n=6) and from patients with no PAD undergoing saphenous vein harvesting for CABG surgery (n=6). We carried out western blot analyses and immunofluorescence to study the TLR expression, activation and distribution in human skeletal muscle biopsy. **Results:** Western blot analyses on muscle homogenates showed upregulation of TLR4&1 in CLI ($P<0.05$); Furthermore, phosphorylated NFkB and JNK as key known signaling pathways were found to be increased in ischaemic samples ($P<0.05$) [Figure1]. TLR4&1 double staining of ischaemic muscle sections demonstrated co-localization of these receptors, suggestive of heterodimerisation. Also, we demonstrated TLR1 is expressed on endothelium (CD31), neutrophils (CD43) and macrophages (CD68) in CLI. **Conclusions:** We showed that TLR4&1 are upregulated in

CLI. Also, Our study suggests that upregulated TLR4&1 are functional in CLI. Further studies are required to understand the downstream signalling, which may lead to the development of novel therapies.

Expression and Distribution of Toll-like Receptor 4 and 1 in Ischaemic Skeletal Muscle

Ali Navi, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Sidney G. Shaw**, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **Janice Tsui**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK;

Purpose: It has been suggested that the inflammation induced by ischaemia in peripheral arterial disease (PAD) has a key role in damaging lower limb skeletal muscle. Toll-like receptors (TLRs) are key pattern recognition receptors in the innate immune system. TLR4 has been implicated in ischaemia. Also, TLR4 has been shown to form heterodimers with TLR1 in animal model. We aimed to define the changes in TLR4&1 expression and distribution in human critical limb ischaemia (CLI). **Methods:** We collected human skeletal muscle biopsies from the medial head of gastrocnemius from patients undergoing major lower limb amputation due to CLI (n=6) and from patients with no PAD undergoing saphenous vein harvesting for CABG surgery (n=6). We carried out western blot analyses and immunofluorescence to study the TLR expression and distribution in human skeletal muscle biopsy respectively. **Results:** Western blot analyses on muscle homogenates showed upregulation of TLR4&1 in CLI ($P<0.05$) [Figure 1]. TLR4 and TLR1

double staining of ischaemic muscle sections demonstrated co-localization of these receptors, suggestive of TLR4&1 heterodimerisation. Furthermore, we demonstrated that the TLR1 is expressed on endothelium (CD31), neutrophils (CD43) and macrophages (CD68) in CLI. **Conclusions:** We showed that TLR4&1 are upregulated in CLI. Further studies are required to understand the downstream signalling, which may lead to the development of novel therapies aimed at reducing muscle damage.

Downstream Signaling Pathway of Toll-like Receptor 4 and 1 in Ischaemic Skeletal Muscle

Ali Navi, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Sidney G. Shaw**, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **Janice Tsui**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK;

Purpose: The inflammation induced by ischaemia in peripheral arterial disease (PAD) has significant role in damaging lower limb skeletal muscle. Toll-like receptors (TLRs) are key pattern recognition receptors in the innate immune system. Whilst the upregulation of TLR4&1 has been shown in critical limb ischaemia (CLI), the activation of the related transcription factors has not been demonstrated. We aimed to identify the downstream signalling mechanisms contributing to cellular damage in ischaemic human skeletal muscle. **Methods:** We collected human skeletal muscle biopsies from the medial head of gastrocnemius from patients undergoing major lower limb amputation due to CLI (n=4) and from patients with no PAD undergoing

saphenous vein harvesting for CABG surgery (n=4). We carried out western blot analyses to quantify the amount of transcription factors in the human muscle biopsies. **Results:** Western blot analyses on muscle homogenates showed increased phosphorylated NFkB and phosphorylated JNK in CLI ($P<0.05$). Furthermore, western blot analyses of cleaved caspase 3 expression was also performed to measure apoptosis within the muscle biopsies and this was found to be increased in the ischaemic samples ($P<0.05$) [Figure 1]. **Conclusions:** Our results suggest that both NFkB and JNK are activated in CLI. Further studies are required to establish the link between TLR4&1 and these transcription factors, which may lead to develop novel therapies aimed at reducing muscle damage.

Functional consequences of ischaemia-induced Toll-like Receptor 4 (TLR4) activation in skeletal muscle

Ali Navi, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **Hemanshu Patel**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK, **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Daryll M. Baker**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **Janice Tsui**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK;

Objectives: Inflammation contributes to ischaemia-induced myopathy in patients with peripheral arterial disease (PAD). TLR4 is a well-recognized pattern recognition receptor that has been implicated in ischaemia-induced tissue injury. We hypothesize that TLR4 is upregulated and its signalling pathway activated in cultured human myotubes exposed to simulated ischaemia. **Methods:** Human gastrocnemius muscle biopsies were taken

from patients with critical limb ischaemia undergoing major lower limb amputation (Group 1) and from patients with no PAD (Group 2). Human myoblasts were isolated, cultured to myotubes and then pre-treated with TLR4 neutralizing antibody prior to exposure to simulated ischaemia. Western blot analysis of TLR4, P-NFkB (signal-related kinase) and cleaved caspase III were carried out on the cell lysates to assess TLR4 expression, downstream signalling activity and apoptosis respectively. **Results:** Upregulation of TLR4, P-NFkB and cleaved caspase III was observed after simulated ischaemia in both groups ($P<0.05$). Further, pre-treatment with TLR4 neutralizing antibody prior to simulated ischaemia reduced the expression of P-NFkB and cleaved caspase III in ischaemic cell lysates as compared to ischaemic cell lysates with no pre-treatment ($P<0.05$). **Conclusion:** TLR4 is upregulated and its downstream signalling pathway is activated in the ischaemic human myotubes *in vitro*. Further, inhibition of TLR4 prior to ischaemia was associated with inhibition of the signalling pathway and reduced ischaemia-induced apoptosis. Further studies are required to understand the downstream signaling, which may lead to the development of novel therapies aimed at reducing muscle damage in patients with CLI.

Emerging role of Toll-like Receptor 4 and Heat Shock Protein 60&70 in ischaemia-induced skeletal muscle damage *in vitro*

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OBJECTIVES: The innate immune response contributes to the skeletal muscle damage in patients with critical limb ischaemia (CLI); however, the detailed signaling mechanisms are not fully understood. We hypothesized that simulated ischaemia induces inflammatory cytokine release from skeletal

myotubes, via a mechanism that involves heat shock protein (HSP) 60&70, known endogenous ligands of Toll-like receptor 4 (TLR4), in vitro. **METHODS:** Human gastrocnemius muscle biopsies were taken from patients with CLI undergoing major lower limb amputation and from patients with no peripheral arterial disease (PAD). Human myoblasts were isolated, cultured to myotubes and then pre-treated with TLR4 neutralizing antibody prior to exposure to simulated ischaemia. Fluorescent immunostaining was carried out to confirm cell differentiation; ELISA analysis were carried out to quantify IL6 and TNFα release; and Western blot was used to assess expression of HSP60&70, TLR4 and cleaved caspase-3 as a marker of apoptosis. **RESULTS:** Myotubes from patients with CLI expressed greater levels of cleaved caspase-3 and TLR4 as compared to those from patients with no PAD. When exposed to ischaemic conditions, increased IL6 and TNFα release and upregulation of HSP60&70, cleaved caspase-3 and TLR4 were observed in myotubes from both groups of patients compared to culturing in normoxic conditions ($P<0.05$). Pre-treatment of myotubes from patients with CLI with TLR4 neutralizing antibody prior to simulated ischaemia was associated with reduced expression of HSP60&70, IL6, TNFα and cleaved caspase-3 ($P<0.05$). **CONCLUSIONS:** Increased cytokine release, apoptosis and expression of HSP60&70 and TLR4 occur in ischaemic skeletal muscle in vitro. TLR4 antagonism was associated with reduced apoptosis and inflammatory cytokine release and down-regulation of HSP60&70 expression. This suggests a potential pathway where TLR4 and its endogenous ligands contribute to a positive feedback loop to maintain a proinflammatory environment during ischaemia.

Detrimental role of MyD88-dependent signaling and Toll-like receptor 4 in ischaemia induced skeletal muscle damage in vitro.

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London, UK. [2]Centre for Rheumatology & Connective Tissue Disease, UCL, Royal Free Campus, UK. [3]Department of Clinical Research, University of Bern, Switzerland

Background: Mounting evidence supports a detrimental role of the innate immune system in the pathophysiology of skeletal muscle damage in patients with peripheral arterial disease (PAD); however, the downstream signaling pathway is poorly understood. Toll-like receptor 4 (TLR4) is a well-recognized pattern recognition receptor that has been implicated in ischaemia-induced skeletal muscle damage. We hypothesized that the MyD88 downstream signaling pathway is involved in regulating inflammatory gene transcription in TLR4-induced skeletal muscle damage in ischaemia. **Methods:** Human gastrocnemius muscle biopsies were taken from patients with critical limb ischaemia (CLI) undergoing major lower limb amputation and from patients with no PAD. Human myoblasts were isolated, cultured to myotubes and then pre-treated with MyD88 or TRIF inhibitor prior to exposure to simulated ischaemia. Fluorescent immunostaining was carried out to confirm cell differentiation; ELISA analyses were carried out to quantify IL6 and TNFα release; and Western blot was used to assess expression of TLR4, P-NFkB (transcription protein) and cleaved caspase-3 as a marker of apoptosis. **Results:** Increased IL6 and TNFα release and upregulation of TLR4, P-NFkB and cleaved caspase-3 were observed after simulated ischaemia in myotubes from both groups ($P<0.05$); however, myotubes from patients with CLI expressed greater levels as compared to those from patients with no PAD ($P<0.05$). Pre-treatment of myotubes from patients with CLI with MyD88 inhibitor prior to ischaemia showed decreased IL6 and TNF-α release and down-regulation of cleaved caspase-3 ($P<0.05$). However, myotubes pre-treated with TRIF inhibitor disclosed no significant changes in IL6 and TNFα release or cleaved caspase-3 expression. **Conclusion:** TLR4 is upregulated and its downstream signaling pathway is activated with increased cytokine release and apoptosis in ischaemic skeletal muscle *in vitro*. Inhibition of the

MyD88-dependent signaling pathway constrains inflammatory cytokine production in the presence of ischaemia-induced TLR4-activation. Further studies are required to understand the downstream signaling, which may lead to the development of novel therapies aimed at reducing muscle damage in patients with CLI.

Detrimental role of Heat Shock Protein 60&70 and Toll-like Receptor 4 in skeletal muscle ischaemia *in vitro*

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[3]Department of Clinical Research, University of Bern, Switzerlan

Objectives: The innate immune response contributes to the skeletal muscle damage in patients with critical limb ischaemia (CLI); however, the detailed signalling mechanisms are not fully understood. We hypothesised that simulated ischaemia induces inflammatory cytokine release from skeletal myotubes, via a mechanism that involves heat shock protein (HSP) 60&70, known endogenous ligands of Toll-like receptor 4 (TLR4), *in vitro.* **Methods:** Human gastrocnemius muscle biopsies were taken from patients with CLI undergoing major lower limb amputation and from patients with no peripheral arterial disease (PAD). Human myoblasts were isolated, cultured to myotubes and then pre-treated with TLR4 neutralizing antibody prior to exposure to simulated ischaemia. Fluorescent immunostaining was carried out to confirm cell differentiation; ELISA analysis were carried out to quantify IL6 and TNFα release; and Western blot was used to assess expression of HSP60&70, TLR4 and cleaved caspase-3 as a marker of apoptosis. **Results:** Myotubes from patients with CLI expressed greater levels of cleaved caspase-3 and TLR4 as compared to those from patients with no PAD. When exposed to

ischaemic conditions, increased IL6 and TNFα release and upregulation of HSP60&70, cleaved caspase-3 and TLR4 were observed in myotubes from both groups of patients compared to culturing in normoxic conditions ($P<0.05$). Pre-treatment of myotubes from patients with CLI with TLR4 neutralizing antibody prior to simulated ischaemia was associated with reduced expression of HSP60&70, IL6, TNFα and cleaved caspase-3 ($P<0.05$). **Conclusion:** Increased cytokine release, apoptosis and expression of HSP60&70 and TLR4 occur in ischaemic skeletal muscle in vitro. TLR4 antagonism was associated with reduced apoptosis and inflammatory cytokine release and down-regulation of HSP60&70 expression. Here we report an intimate link between HSP60&70-induced inflammatory cytokine production and TLR4 inhibition in ischaemic human myotubes.

Mouse Model of Ischaemia-induced Skeletal Muscle Damage

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* Joint first authors

Objectives: The role of ischaemia-induced myopathy in the poor functional outcomes of patients with critical limb ischaemia (CLI) is increasingly recognised, however, the detailed mechanisms involved are not fully understood. Accordingly, we aimed to develop a murine model of hind-limb ischaemia which recapitulates the pathophysiological changes seen in human CLI muscle to aid further studies in this area and to evaluate potential novel therapies. **Methods:** Firstly, changes in critically ischaemic human skeletal muscle were assessed in gastrocnemius biopsies taken from patients undergoing major lower limb amputation due to CLI. Histomorphological

changes were assessed using H&E staining. In order to identify a murine model which best recapitulates these changes, hindlimb ischaemia was induced by excision of common and superficial femoral artery and ligation of the side branches in BALB/c and C57BL/6 mice of 6 and 12 weeks of age. Tissue perfusion and evidence of morphological changes and damage within the ischaemic muscle were assessed at days 1, 3, 7, 14 and 21 by laser Doppler and H&E staining, respectively. **Results:** In line with previous reports, we demonstrated features of significant muscle fibre injury in muscle sections from CLI patients compared to non-ischaemic control donors. These included loss of muscle fibres with concomitant fibroadipocytic changes, as well as variation in muscle fibre size and evidence of degeneration and necrosis. Analysis of muscle biopsies from in vivo studies demonstrated a similar pattern of histomorphological changes in both C57BL/6 and BALB/c strains at day 7, 14 and 21. However younger animals showed less severe muscle damage, and faster resolution of muscle injury (resolution by day 21). **Conclusion:** Excision of common and superficial femoral artery with ligation of the side branches in 12 week old mice resulted in reproducible ischaemia-induced skeletal muscle changes that closely mimicked those that occur in human CLI. This provides a useful pre-clinical model for further work including the assessment of novel muscle-protective adjunctive therapies for the treatment of CLI.

Toll-like receptor 4 (TLR4) inhibition reduces skeletal muscle damage in critical limb ischaemia.

Ali Navi, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK**;**

Sidney G. Shaw, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK; **Janice Tsui**, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK;

Objective: The innate immune response has emerging role in skeletal muscle damage in patients with critical limb ischaemia. We have previously shown that TLR4, a well-recognized pattern recognition receptor, is upregulated and functional in cultured human myotubes exposed to simulated ischaemia. We show herein that inhibition of TLR4 enhanced recovery from ischaemia-induced damage in skeletal muscle, *in vivo*. **Methods:** The hind-limb ischaemia was induced by excision of common and superficial femoral arteries in wild-type (WT), endogenously deleted (TLR4-/-) and exogenously inhibited TLR4 (LPS-RS) mice (CL57/BL6, n=18 per group, 12 weeks old), while 9 animals per group were sham operated. The hind-limb tissue and serum samples were collected at day 3, 7 and 21, when laser Doppler and H&E staining were applied to measure haemodynamics and the inflammatory damage. TLR4 mRNA expression and systemic levels of IL-6 & TNFα were also examined by RT-PCR and ELISA, respectively. **Results:** Hind–limb ischaemia of WT mice demonstrated significant up-regulation of TLR4 mRNA expression when compared with sham operated WT mice ($P=0.013$). In addition, marked increase in the level of IL6 & TNFα ($P<0.05$) and slower blood flow recovery ($P<0.05$) were observed in WT mice in comparison with TLR4-/- and LPS-RS groups at day 3, 7 and 21. Further, WT mice displayed more severe ischaemia-induced inflammatory damage in the skeletal muscle after hind-limb ischaemia. **Conclusion:** TLR4 inhibition was associated with significant reduced damage to the skeletal muscle after the ischaemic insult. Here we report an intimate link between TLR4 signalling and ischaemia-induced skeletal muscle damage.

Role of Toll-like receptor 4 (TLR4) inhibition in ischaemia-induced systemic inflammatory response and skeletal muscle damage

Ali Navi, Royal Free London Vascular, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK**; Sidney G. Shaw**, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Royal Free London Vascular, London, UK; **Janice Tsui**, Royal Free London Vascular, London, UK

Introduction: The innate immune response plays an important role in skeletal muscle damage in hind-limb ischaemia, however the mechanism is not fully understood. Previously, we showed less apoptotic changes in cultured human myotubes in simulated ischaemia when TLR4, a pattern recognition receptor, was inhibited. Herein, we investigated whether TLR4 inhibition reduces the ischaemia-induced skeletal muscle damage, via a mechanism that involves systemic IL6 and TNFα, *in vivo*. **Methods**: Hind-limb ischaemia was induced by excision of femoral artery in 12-week-old wild-type (WT) and TLR4-/- mice. Further, a group of CL57/BL6 mice received the TLR4 antagonist, LPS-RS (n=18 per group plus 9 sham operated per group). Laser Doppler was used to measure haemodynamic changes. Tissue collection and serum samples were obtained at day 3, 7 and 21. Systemic levels of IL-6 & TNFα, cleaved caspase-3 (marker of apoptosis) and inflammatory damage were examined by ELISA, Fluorescent IHC-P and H&E staining, respectively. **Results:** TLR4-/- mice and mice given LPS-RS, demonstrated reduced systemic IL6 & TNFα levels ($P<0.05$). This was associated with significant improved blood flow recovery ($P<0.05$), diminished apoptosis and attenuated inflammatory cell infiltration ($P<0.05$) in the skeletal muscle following hind-limb ischaemia as compared to WT mice at day 3, 7 and 21. **Conclusions:** Endogenous deletion and exogenous inhibition of

TLR4 were associated with reduced systemic IL6 & TNFα levels and skeletal muscle damage following hind-limb ischaemia. Here we report an intimate link between ischaemia-induced systemic inflammatory cytokine production and TLR4 inhibition.

Toll-like receptor 4 (TLR4) inhibition reduces ischaemia-induced skeletal muscle damage.

Ali Navi, Royal Free London Vascular, London, UK; David Abraham, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; Xu Shi-Wen, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; Sidney G. Shaw, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; Daryll M. Baker, Royal Free London Vascular, London, UK; Janice Tsui, Royal Free London Vascular, London, UK

Objective: There is an emerging role for the innate immune response in skeletal muscle damage in critical limb ischaemia. We have shown that TLR4, a pattern recognition receptor, is upregulated and functional in cultured human myotubes exposed to simulated ischaemia. This study investigates the effect of TLR4 inhibition on ischaemia-induced skeletal muscle damage *in vivo*. **Methods:** Hindlimb ischaemia was induced by excision of femoral artery in 12 week old wild-type (WT) and TLR4-/- mice. In addition, a group of CL57/BL6 mice received the TLR4 antagonist LPS-RS (n=18 per group plus 9 sham operated per group). Laser Doppler was used to measure haemodynamic changes. Tissue collection and serum samples were obtained at day 3, 7 and 21. TLR4 mRNA expression, systemic levels of IL-6 & TNFα and inflammatory damage were examined by RT-PCR, ELISA and H&E staining, respectively. **Results:** TLR4 mRNA was upregulated in ischaemic skeletal muscle of WT mice ($P<0.05$ compared to sham operated controls). TLR4-/- mice and mice given LPS-RS demonstrated improved blood flow

recovery (P<0.05), reduced systemic IL6 & TNFα levels (P<0.05) and attenuated histological evidence of ischaemia-induced inflammation following hindlimb ischaemia as compared to WT mice at days 3, 7 and 21. **Conclusion:** TLR4 upregulation occurs in ischaemic skeletal muscle *in vivo*. Furthermore, endogenous deletion and exogenous inhibition of this receptor were associated with reduced skeletal muscle damage and systemic inflammatory markers following hindlimb ischaemia, implicating its role in ischaemic skeletal muscle damage.

Critical Role of Toll-like Receptor 4 (TLR4) in Ischaemia-induced Skeletal Muscle Damage

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[2]Centre for Rheumatology & Connective Tissue Disease, UCL, Royal Free Campus, UK

Objectives: Inflammation contributes to skeletal muscle damage in patients with critical limb ischaemia (CLI), in whom patient oriented functional outcomes are poor. Previously, we showed that TLR4, a pattern recognition receptor, is upregulated and activated in skeletal muscle of patients with CLI. This study aimed to investigate whether the TLR4 pathway contributes to skeletal muscle damage in CLI, using *in vitro* and *in vivo* models. **Methods:** Human myoblasts, isolated from skeletal muscle biopsies, were cultured to myotubes and exposed to simulated ischaemia, with or without TLR4 inhibitor pre-treatment. Western blot analyses of P-NFkB (signal-related kinase) and cleaved caspase-3 were carried out on cell lysates to assess downstream signalling activity and apoptosis respectively. Further, hind-limb ischaemia by excision of femoral artery was induced in 12-week old TLR4-/- and wild-type (WT) mice with and without administration of the TLR4 antagonist, LPS-RS (n=18 per group). Systemic levels of IL6 & TNFα, tissue perfusion,

inflammatory damage and apoptosis within the ischaemic muscle were assessed at days 3, 7 and 21 by ELISA, laser Doppler, H&E staining and Fluorescent IHC-P, respectively. **Results:** Simulated ischaemia in cultured human myotubes was associated with upregulation of TLR4, P-NFkB and cleaved caspase-3. Pre-treatment with TLR4 inhibitor reduced P-NFkB and cleaved caspase-3 expression ($P<0.05$). In *in vivo* studies, TLR4-/- mice and mice given LPS-RS exhibited reduced systemic IL6 & TNFα levels ($P<0.05$) following hind-limb ischaemia. This was associated with improved tissue perfusion recovery ($P<0.05$), reduced inflammatory cell infiltration and diminished apoptosis ($P<0.05$) in the ischaemic limb as compared to untreated WT mice at days 3, 7 and 21. **Conclusion:** TLR4 inhibition prior to ischaemia in human myotubes *in vitro*, was associated with inhibition of the signalling pathway and reduced ischaemia-induced apoptosis. Further, endogenous deletion and exogenous inhibition of TLR4 *in vivo* were associated with reduced systemic IL6 & TNFα levels and skeletal muscle damage following hind-limb ischaemia. These findings suggest that TLR4 plays a key role in ischaemia-induced skeletal muscle damage.

Role of Toll-like receptor 4 (TLR4) inhibition in ischaemia-induced systemic inflammatory response and skeletal muscle damage

Ali Navi, Royal Free London Vascular, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK**; Sidney G. Shaw**, Department of Clinical Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Royal Free London Vascular, London, UK; **Janice Tsui**, Royal Free London Vascular, London, UK

Introduction: The innate immune response plays an important role in skeletal muscle damage in hind-limb ischaemia, however the mechanism is

not fully understood. Previously, we showed less apoptotic changes in cultured human myotubes in simulated ischaemia when TLR4, a pattern recognition receptor, was inhibited. Herein, we investigated whether TLR4 inhibition reduces the ischaemia-induced skeletal muscle damage, via a mechanism that involves systemic IL6 and TNFα, *in vivo*. **Methods**: Hind-limb ischaemia was induced by excision of femoral artery in 12-week-old wild-type (WT) and TLR4-/- mice. Further, a group of CL57/BL6 mice received the TLR4 antagonist, LPS-RS (n=18 per group plus 9 sham operated per group). Laser Doppler was used to measure haemodynamic changes. Tissue collection and serum samples were obtained at day 3, 7 and 21. Systemic levels of IL-6 & TNFα, cleaved caspase-3 (marker of apoptosis) and inflammatory damage were examined by ELISA, Fluorescent IHC-P and H&E staining, respectively. **Results:** TLR4-/- mice and mice given LPS-RS, demonstrated reduced systemic IL6 & TNFα levels ($P<0.05$). This was associated with significant improved blood flow recovery ($P<0.05$), diminished apoptosis and attenuated inflammatory cell infiltration ($P<0.05$) in the skeletal muscle following hind-limb ischaemia as compared to WT mice at day 3, 7 and 21. **Conclusions:** Endogenous deletion and exogenous inhibition of TLR4 were associated with reduced systemic IL6 & TNFα levels and skeletal muscle damage following hind-limb ischaemia. Here we report an intimate link between ischaemia-induced systemic inflammatory cytokine production and TLR4 inhibition.

Toll-like receptor 4 (TLR4) inhibition reduces ischaemia-induced skeletal muscle damage.

Ali Navi, Royal Free London Vascular, London, UK; **David Abraham**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK; **Xu Shi-Wen**, Centre for Rheumatology and Connective Tissue Disease, University College London, Royal Free Campus, London, UK**; Sidney G. Shaw**, Department of Clinical

Experimental Research, University of Bern, 3010 Bern, Switzerland; **Daryll M. Baker**, Royal Free London Vascular, London, UK; **Janice Tsui**, Royal Free London Vascular, London, UK

Objective: There is an emerging role for the innate immune response in skeletal muscle damage in critical limb ischaemia. We have shown that TLR4, a pattern recognition receptor, is upregulated and functional in cultured human myotubes exposed to simulated ischaemia. This study investigates the effect of TLR4 inhibition on ischaemia-induced skeletal muscle damage *in vivo*. **Methods**: Hindlimb ischaemia was induced by excision of femoral artery in 12 weeks old wild-type (WT) and TLR4-/- mice. In addition, a group of CL57/BL6 mice received the TLR4 antagonist LPS-RS (n=18 per group plus 9 sham operated per group). Laser Doppler was used to measure haemodynamic changes. Tissue collection and serum samples were obtained at day 3, 7 and 21. TLR4 mRNA expression, systemic levels of IL-6 & TNF-α and inflammatory damage were examined by RT-PCR, ELISA and H&E staining, respectively. **Results:** TLR4 mRNA was upregulated in ischaemic skeletal muscle of WT mice ($P<0.05$ compared to sham operated controls). TLR4-/- mice and mice given LPS-RS demonstrated improved blood flow recovery ($P<0.05$), reduced systemic IL6 & TNFα levels ($P<0.05$) and attenuated histological evidence of ischaemia-induced inflammation following hindlimb ischaemia as compared to WT mice at days 3, 7 and 21. **Conclusion:** TLR4 upregulation occurs in ischaemic skeletal muscle *in vivo*. Furthermore, endogenous deletion and exogenous inhibition of this receptor were associated with reduced skeletal muscle damage and systemic inflammatory markers following hindlimb ischaemia, implicating its role in ischaemic skeletal muscle damage.

5. Patient information sheet, consent form and data collection form

Royal Free and University College Medical School
UNIVERSITY COLLEGE LONDON
Address for Correspondence:
University Department of Surgery
Royal Free Campus
The Royal Free Hospital
Pond Street,
London NW3 2QG
Telephone: 020 77940500
Facsimile: 02074726711

PATIENT INFORMATION SHEET

Study Title: A study of muscle injury in peripheral vascular disease

You are invited to participate in a research study. This information sheet explains why we are conducting this study and how it is being done. Please take some time to read the information carefully and to decide whether you would like to take part or not. Please discuss it with your family and friends as you wish and ask us if you have any questions.

What is the purpose of this study?

The aim of this project is to study some aspects of peripheral vascular disease which are not well understood. Peripheral vascular disease is a common condition where the blood supply to the legs is impaired and the leg muscles are damaged. At present, treatment includes bypass surgery to improve the circulation. We aim to study the mechanisms that are involved in causing muscle damage secondary to peripheral vascular disease. A better understanding of these mechanisms may improve treatment for this disease in the future.

Why have I been chosen?

You have been invited to take part either because you suffer from peripheral vascular disease or because the operation you will be undergoing is similar in many ways to that done for peripheral vascular disease. This allows us to collect suitable samples for analyses and make appropriate comparisons.

Do I have to take part?

You do not have to take part in this study if you do not want to. If you decide to take part you may withdraw at any time without giving a reason. Your decision to take part or not will not affect your care in any way.

What will happen to me if I take part?

Up to 2 calf muscle samples, each the size of a small pea, will be taken during the operation. These are for the study and not part of the treatment. None of these should cause any ill effect or additional discomfort and will not affect your recovery from your operation.

What do I have to do?

You do not have to do anything more than you need to for your operation.

What are the possible disadvantages and risks in taking part?

You will have small samples of your calf muscle taken. However, these will be done during the operation when you are asleep and so will not cause any discomfort. Your treatment and recovery will not be affected.

What are the possible benefits of taking part?

This study will help us improve treatment for this condition in the future. However, at this stage, there will be no immediate benefits to you.

What if something goes wrong?

This study does not involve any extra treatment or any high-risk procedures so nothing is likely to go wrong. However, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

Will taking part in this study be kept confidential?

Any information collected about you during the course of the research will be

kept strictly confidential.

What happens to the results of the research study?

Results from this study will be published in a medical journal in approximately a year's time. However you will not be identified in any report or publication. If you wish to know the outcome of the study, you can do so by contacting us.

Who is organizing and funding the research?

This study is organised and funded by the Vascular Unit of the Department of Surgery.

Who has reviewed the study?

The Research Ethics Committee of the Royal Free Hospital has reviewed this study. If you have any questions or queries at any stage, please contact:

Miss Janice Tsui
University Department of Surgery
Royal Free Hospital
Pond Street
London NW3 2QG
Tel: 020-7794-0500 Ext 33938

You will be given a copy of the information sheet and a signed consent form to keep. Thank you very much for taking part in this study.

Consent Form

Project Title: Study of Muscle Injury in Peripheral Vascular Disease

Researcher: Miss Janice Tsui & _____

1. I confirm that I have read and understood the information sheet provided for the above study and have had the opportunity to ask questions.
2. I confirm that I have had sufficient time to consider whether or not I want to be included with the study
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reasons, without my medical care or legal rights being affected.
4. I understand that sections of my medical notes may be looked at by responsible individuals from NHS or from regulatory authorities where it is relevant to my taking part in the research. I understand that samples of my muscle may be kept up to 6 months for other studies.
5. I agree to take part in the above study

Name of patient Date

Signature

Name of person taking consent Date

Signature

Patient data:

Sample Type:
Normal or Ischaemic

Reference Number:

Information sheet/ Consent:

Hospital Number:

Ethnic Group:

Name:

Sex:

Date of Birth:

Date of onset:

PMH:

Angiogram:

ECG:

Bleeding Disorders:

Diabetes:

IHD:

CVA:

Hypertension

Other:

Medications:

Smoking Hx

Investigations:

ABPI:

Duplex :

Fontaine Classification:

Coronary Angiogram

6. Ethics approval and personal licence

Royal Free & Medical School Local Research Ethics Committee

NHS

Please reply to:

Ethics Department
Royal Free Hospital
Pond Street
London
NW3 2QG

Telephone: 020 7830 2746
Fax: 020 7830 2961
E-mail: rosemary.brown@royalfree.nhs.uk

21 May 2010

Mr Daryll M Baker,
University Department of Vascular Surgery
Royal Free Hampstead NHS Trust
Pond Street
Hampstead
London
NW3 2QG

Dear Mr Baker,

REC Ref: 29-2000 **Changes in ischaemic muscle and lower limb arteries in perpheral arterial disease**

Thank you for your letter dated 18th May 2010 reporting on the progress of this study.

Your request to continue with the trial has been approved by Chairman's Action

Therefore, you are free to continue with your project.

Yours sincerely

Rosemary Brown
Ethics Committee Adviser

I95F72597
26 August 2014

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Home Office

Animals in Science Regulation Unit
Home Office Science
Mail Point B20,
4th Floor, Seacole Building
2 Marsham Street, London SW1P 4DF
Tel 0207 035 0477
Web Site http://www.homeoffice.gov.uk/

Dr S Hosseini Navi
The Registered Department of the Royal Free and University College Medical School, Royal Free Campus, and the Royal Free Hospital
University College London Medical School
CBU, Royal Free Campus
Rowland Hill Street
Hampstead
London
NW3 2PF

Our Reference: I95F72597

Date: 26 August 2014

UNIVERSITY COLLEGE LONDON
ROYA CAMPUS
CBU
Duncan Moore
DATE: 5/9/14

Dear Dr Hosseini Navi

ANIMALS (SCIENTIFIC PROCEDURES) ACT 1986

Please find attached your personal licence.

You should check through your licence carefully for any endorsements on this licence in relation to animal types, licence category and the conditions attached to it. A personal licence on its own does not authorise you to perform regulated procedures on protected animals. You may perform only the procedures of the category specified by it if the procedure is applied as part of a programme of work specified in a project licence authorising the application, as part of that programme, of a regulated procedure of that description to an animal of that description. The application of unauthorised regulated procedures is a contravention of the Act and may result in prosecution and/or variation, suspension or revocation of your licence.

You are required to keep a record of all regulated procedures that you have carried out. This information must be made readily available to the Inspector or Secretary of State when required. If you cease to carry out work requiring a licence (for example leaving the UK to work abroad) you must return your licence to the Home Office.

As soon as you cease to work at the establishment given as the primary availability on your licence, or it ceases to be the place where you wish your licence to be primarily available, you must notify the Home Office, as this change will affect the fees charged. No other person may perform, either in whole or in part, any procedure authorised by your licence. The only exceptions are certain specific tasks of a non-technical nature. No other delegation is permitted (see Guidance on the Operation of the Act).

Should you wish any part of this licence to be amended, you must apply to the Home Office via your Home Office Liaison Contact giving details of the changes requested.

Yours sincerely

ASRU Licensing Team

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I95F72597
26 August 2014

Home Office

No. I95F72597

ANIMALS (SCIENTIFIC PROCEDURES) ACT 1986

PERSONAL LICENCE

to

carry out regulated procedures on living animals.

In pursuance of the powers vested in him by the above Act, the Secretary of State hereby licenses

Dr S Hosseini Navi
The Registered Department of the Royal Free and University College Medical School, Royal Free Campus, and the Royal Free Hospital
University College London Medical School
CBU, Royal Free Campus
Rowland Hill Street
Hampstead
London
NW3 2PF

to apply regulated procedures of the category or categories specified below to animals of the species or groups specified below at places specified in authorised project licences subject to the restrictions and provisions contained in the Act, and subject also to the limitations and conditions contained in this licence and to such other conditions as the Secretary of State may from time to time prescribe:

Description of animal(s)

- Guinea-pigs
- Hamsters
- Mice
- Rabbits
- Rats

Categories of regulated procedure:

A. Minor/minimally invasive procedures not requiring sedation, analgesia or general anaesthesia
B. Minor/minimally invasive procedures involving sedation, analgesia or brief general anaesthesia
   Plus - surgical procedures conducted under brief non-recovery general anaesthesia
C. Surgical procedures involving general anaesthesia
   Plus - administration and maintenance of balanced or prolonged general anaesthesia

This licence shall be in force until revoked by the Secretary of State and shall be periodically reviewed by him.

Home Office
2 Marsham Street
London SW1P 4DF

For the Secretary of State

26 August 2014

8b65967b-9d9b-42d9-808d-cacb6737b270

I95F72597
26 August 2014

NB. This licence does not authorise the licensee to perform a regulated procedure to an animal unless the procedure is applied as part of a programme of work specified in a project licence authorising the application, as part of that programme, of a regulated procedure of that description to an animal of that description.

8a65967b-9d9b-42d9-808d-cacb6737b270

Home Office

No. I95F72597
26 August 2014

Personal Licence - Additional Conditions

Any additional conditions that apply to this licence are set out below -

- None.

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7. Oxford Centre for Evidence-based Medicine – Levels of Evidence (March 2009)

Level	Therapy / Prevention, Aetiology / Harm	Prognosis	Diagnosis	Differential diagnosis / symptom prevalence study
1a	SR (with homogeneity) of RCTs	SR (with homogeneity) of inception cohort studies; CDR validated in different populations	SR (with homogeneity) of Level 1 diagnostic studies; CDR with 1b studies from different clinical centres	SR (with homogeneity) of prospective cohort studies
1b	Individual RCT (with narrow Confidence Interval)	Individual inception cohort study with > 80% follow-up; CDR validated in a single population	Validating cohort study with good reference standards; or CDR tested within one clinical centre	Prospective cohort study with good follow-up
1c	All or none	All or none case-series	Absolute SpPins and SnNouts	All or none case-series
2a	SR (with homogeneity) of cohort studies	SR (with homogeneity) of either retrospective cohort studies or untreated control groups in RCTs	SR (with homogeneity) of Level >2 diagnostic studies	SR (with homogeneity) of 2b and better studies
2b	Individual cohort study (including low quality RCT; e.g., <80% follow-up)	Retrospective cohort study or follow-up of untreated control patients in an RCT; Derivation of CDR or validated on split-sample only	Exploratory cohort study with good reference standards; CDR after derivation, or validated only on split-sample or databases	Retrospective cohort study, or poor follow-up
2c	“Outcomes” Research; Ecological studies	“Outcomes” Research		Ecological studies
3a	SR (with homogeneity) of case-control studies		SR (with homogeneity) of 3b and better studies	SR (with homogeneity) of 3b and better studies

3b	Individual Case-Control Study		Non-consecutive study; or without consistently applied reference standards	Non-consecutive cohort study, or very limited population
4	Case-series (and poor quality cohort and case-control studies)	Case-series (and poor quality prognostic cohort studies)	Case-control study, poor or non-independent reference standard	Case-series or superseded reference standards
5	Expert opinion without explicit critical appraisal, or based on physiology, bench research or “first principles”	Expert opinion without explicit critical appraisal, or based on physiology, bench research or “first principles”	Expert opinion without explicit critical appraisal, or based on physiology, bench research or “first principles”	Expert opinion without explicit critical appraisal, or based on physiology, bench research or “first principles”

Produced by Bob Phillips, Chris Ball, Dave Sackett, Doug Badenoch, Sharon Straus, Brian Haynes, Martin Dawes since November 1998. Updated by Jeremy Howick March 2009.

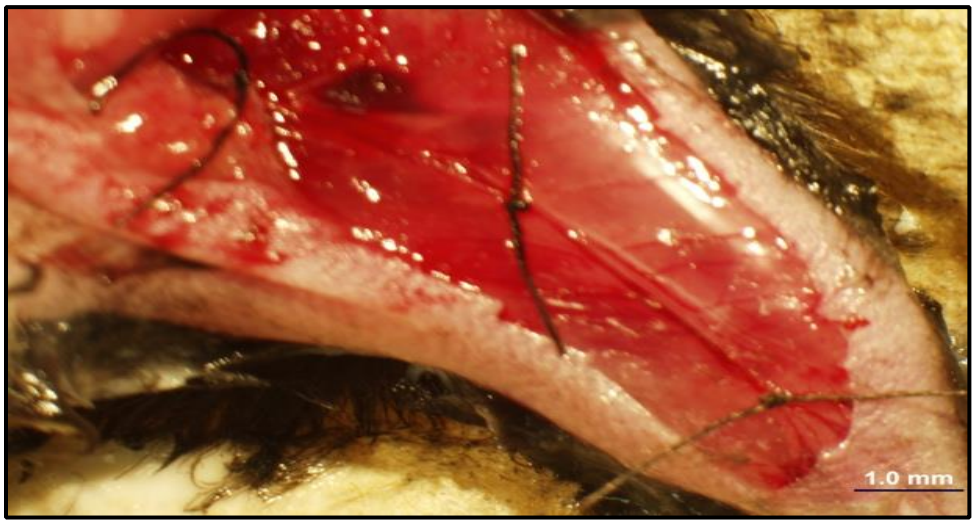

Figure 85 - Demonstrating the sites of ligations in Dardik's model of hind-limb ischaemia

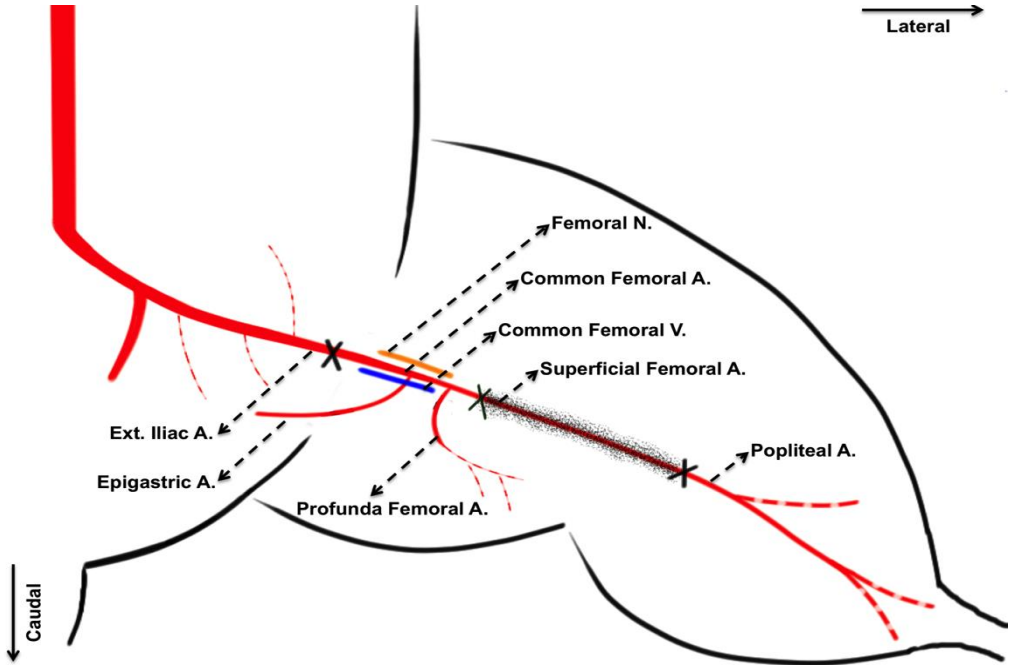

Figure 86 - Dardik model of hind-limb ischaemia

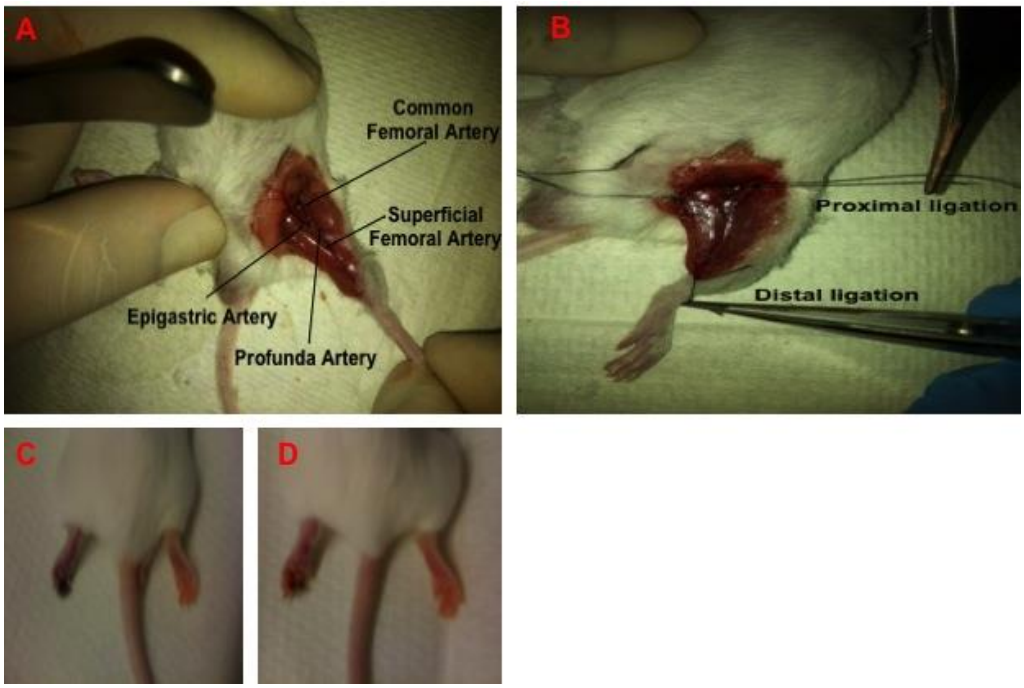

Figure 87 - A) Demonstrating the anatomy of the mouse hind limb, supine position. Extensive dissection of the skin and fascia is for the purpose of demonstration. Common femoral artery appears below the inguinal ligament and gives off two main branches of epigastric and profunda before it forms superficial femoral artery. B) Demonstrating the anatomy of the left mouse hindlimb after ligation of the femoral artery at the proximal and distal sites before the excision of the superficial femoral artery. Tissue necrosis on C) day 7, D) Day 14 after surgery in 11 weeks old male BALB/c mouse.

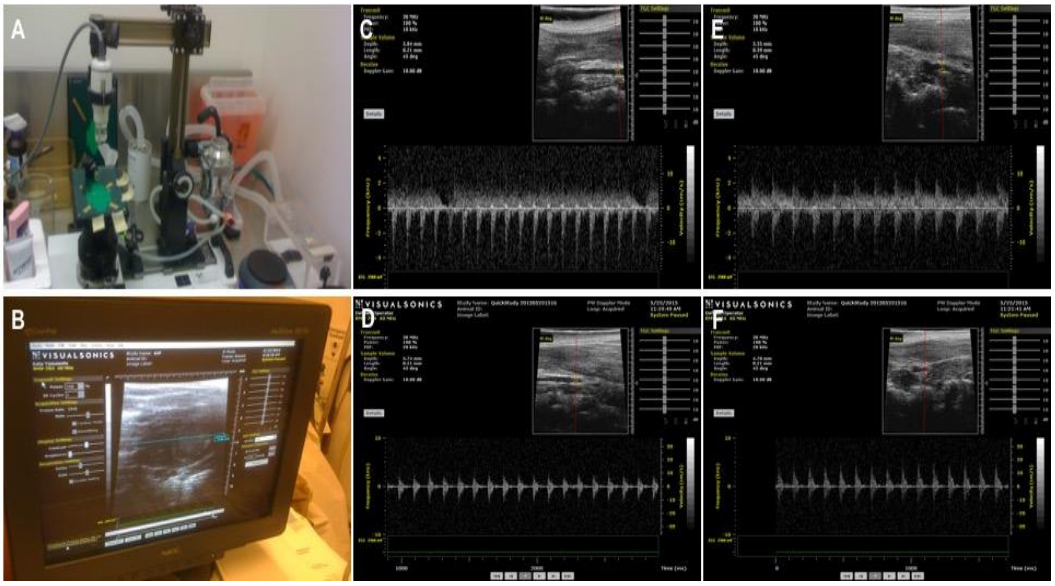

Figure 88 - A, B) Duplex machine with the probe suitable for very small vessels; C,D) Duplex images of proximal and distal aorta; E,F) Duplex images of common iliac artery

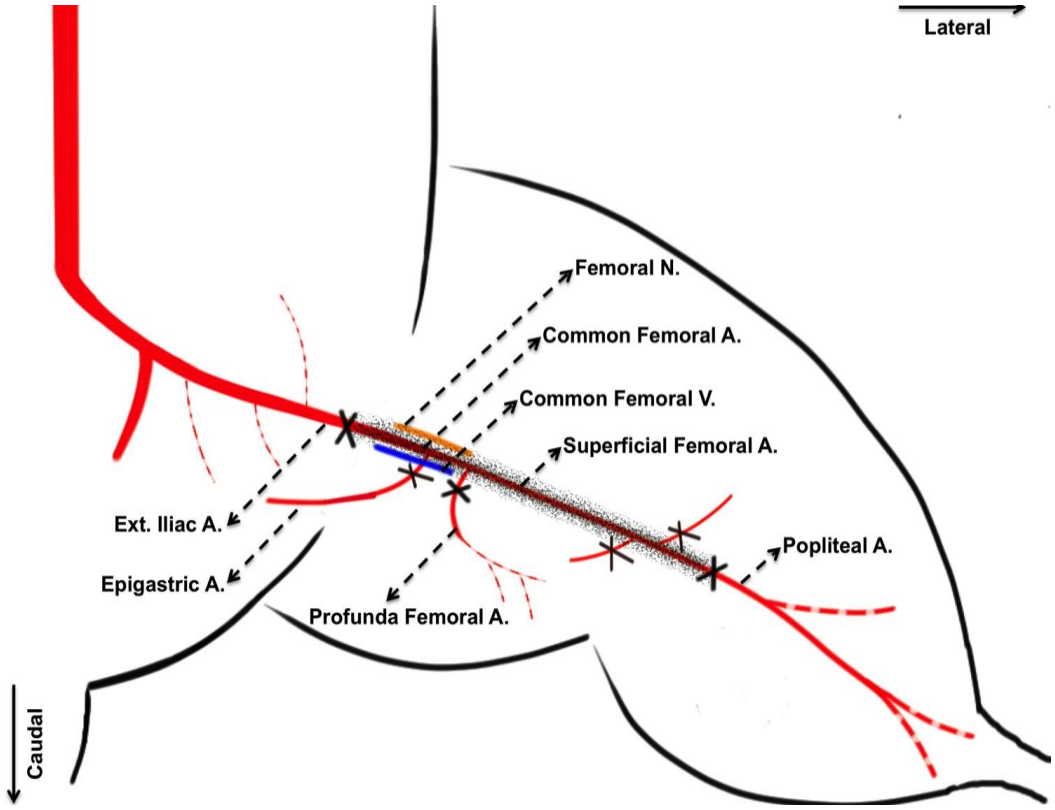

Figure 89 - Messina model of acute limb ischaemia

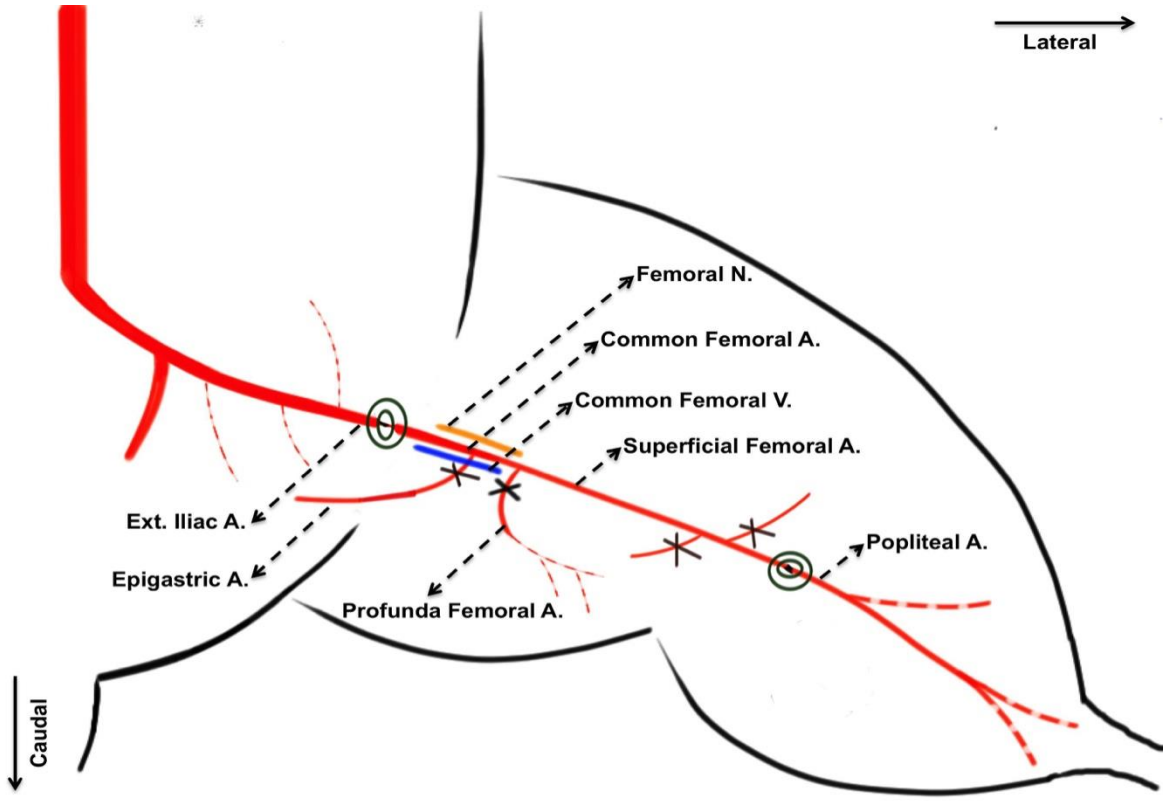

Figure 90 - Messina model of chronic limb ischaemia

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